DEVELOPMENT OF MICROSATELLITE MARKERS TO EVALUATE CURRENT SPECIES BOUNDARIES BETWEEN *Liatris helleri* PORTER AND *Liatris turgida* (GAISER) (ASTERACEAE)

A Thesis
by
LOGAN C. CLARK

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

May 2019
Department of Biology

DEVELOPMENT OF MICROSATELLITE MARKERS TO EVALUATE CURRENT SPECIES BOUNDARIES BETWEEN *Liatris helleri* PORTER AND *Liatris turgida* (GAISER) (ASTERACEAE)

A Thesis
by
LOGAN CLARK
May 2019

APPROVED BY:

Matt C. Estep
Chairperson, Thesis Committee

Jennifer Rhode Ward
Member, Thesis Committee

Howard S. Neufeld
Member, Thesis Committee

Zack E. Murrell
Chairperson, Department of Biology

Mike J. McKenzie, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

DEVELOPMENT OF MICROSATELLITE MARKERS TO EVALUATE CURRENT SPECIES BOUNDARIES BETWEEN *Liatris helleri* PORTER AND *Liatris turgida* (GAISER) (ASTERACEAE)

Logan C. Clark
B.A., Appalachian State University
M.A., Appalachian State University

Chairperson: Matt C. Estep

Liatris helleri Porter (Asteraceae), Heller's blazing star, is a rare perennial herb endemic to high elevation rock outcroppings in the Southern Appalachians. This species has a showy spiked inflorescence with purple flowers that is easily noticed during its flowering period of late July through September. There are less than 10 extant populations of *L. helleri* known throughout western North Carolina, all within a 30 km radius of each other (Godt and Hamrick, 1995). Botanists have historically relied on its short stature, occurrence in high elevation rock outcrop communities, and its short pappus length to distinguish this species from its congener, *Liatris turgida* Gaiser (Nesom, 2005b). *L. turgida*, the shale barren blazing star, is a lower elevation species that displays a similar and sometimes overlapping morphology to that of *L. helleri*. The separation of these two taxa as individual species has been historically contentious with previous studies of genetic diversity and morphology suggesting the possibility that together they might represent a single species with an expanded range.

The genus *Liatris* is composed of approximately 40-50 species located predominately along the eastern seaboard of North America. Broad species ranges in this genus can sometimes lead to polyspecies sympatry where three or more species coexist. This co-occurrence can lead to hybridization between species on rare occasions and a lack of well-defined ecological differentiation

(Levin, 1967). *Liatris* is a genus known to display “unusual difficulty” in specific determination likely due to these occurrences of hybridization and phenotypic plasticity (Gaiser, 1946).

Previous genetic studies in this species employed lower resolution markers such as allozymes and cpDNA markers (Godt and Hamrick, 1996; Sullins 2013). Allozyme studies found highly structured populations, with high genetic diversity. The phylogeographic approach used by Sullins (2013) employed cpDNA markers, which provided no clear separation of *L. helleri* and *L. turgida*, resulting in incomplete lineage sorting. The current study involves the development of a series of 17 microsatellite markers, which were then employed to investigate the genetic diversity, population structure, and genetic distance between these two taxa.

To accomplish this, 21 populations and 327 individuals across the range of *L. helleri* proposed by Nesom (2005b) were sampled and genotyped using 12 of the microsatellite markers developed for this study. This study identifies a genetically distinct metapopulation of *L. helleri*, distinct populations of *L. turgida*, and admixed populations. Population structure within *L. turgida* was also identified and baseline genetic diversity statistics for both species were calculated. The results from this study in conjunction with previous studies offer a more comprehensive understanding toward our concept of *L. helleri* and provide genetic data to inform management decisions. Based on the findings generated in this study *L. helleri* should retain its species distinction separable from *L. turgida*, at least at a subspecies level.

Acknowledgments

I'd like to thank Dr. Matt Estep for all of his help, guidance, and support over the past four years throughout my undergraduate and graduate years. I would not have gotten to this point in my academic career without his encouragement and I am forever grateful. I'd also like to thank my committee members Dr. Jennifer Rhode Ward and Dr. Howard Neufeld for their valuable insight as well as their unwavering help and support through this process. The US Fish and Wildlife Service contributed greatly through the funding of this project. I'd also like to thank my family and friends for all of their support throughout my time at Appalachian State.

Table of Contents

List of Tables

List of Figures

Foreword

Chapter 1 and the Addendum of this thesis have been formatted according to APA guidelines.

Chapter 2 of this thesis has been accepted for publication by *Applications in Plant Science*, an open-access, peer-reviewed journal published by The Botanical Society of America; it has been formatted according to the style guide for that journal.

Chapter 3 of this thesis will be submitted to *Conservation Genetics*, a peer-reviewed journal published by Springer Science and Business Media; it has been formatted according to the style for that journal.

Chapter 1

Introduction

The Genus Liatris

The North American genus *Liatris* (Gaertner ex. Schreber) is composed of 40-50 species mainly confined to the eastern seaboard of North America. The genus is morphologically characterized broadly by a cormose habit, alternate leaves, rose-purple corollas, long corolla tubes, and usually spiciform to racemiform inflorescences (Nesom, 2005a). Most species grow in warm and sandy places, so the genus is most abundant in Texas and Florida. There is one species that occurs in the Bahamas (*L. garberi* A. Gray), several Great-Plains natives, and a single species that occurs in Mexico (*L. punctata*, Hook.) (Gaiser, 1946; Nesom, 2005a; Weakley, 2015). Hybridization and morphological plasticity within *Liatris* has led to challenging descriptions of species and subsequently has led to this genus being considered as one of "unusual difficulty", with unclear delineation of species boundaries for some taxa (Gaiser, 1946).

Cytogenetic work has demonstrated that *Liatris* has small and morphologically indistinct chromosomes with a base chromosome number of n=10 (Gaiser, 1950). Triplication of certain chromosomes has been observed in hybrids of *L. aspera* x *L. spicata* (Levin, 1968). Gaiser (1949, 1950) separated *Liatris* into four series: Graminifoliae, Pauciflorae, Spicatae, and Pycnostachyae. The Graminifoliae series is hypothesized to represent the most primitive form in the genus. The center of origin for this series may be the Appalachian Highlands due the area's biogeographic history and the variety of species in the Graminifoliae series found there. The short and slender stature typical of the series could also have given rise to the taller species found in more western and northern parts of the genus range (Gaiser, 1950). These cytological studies suggest that the primary driver of speciation in this genus is not chromosome (genome) evolution, but instead likely related to ecological adaptations determining species distribution. The species of the Pauciflorae and Spicatae series displayed small chromosomes that closely approximated one another with differences of no more than one pair of chromosomes between the species of the series. Irregular meiosis was observed in *L. acidota* of the

Spicatae series and two collections of *L. pychnostachya* displayed very small additional chromosomes that did not display any noticeable effect. Hybridization in these species could be facilitated by similar chromosome morphology allowing easy formation of homologous pairs (Gaiser, 1949). Species of the Graminifoliae and Pauciflorae display consistent chromosome number and regular meiotic division.

Infrageneric classification of *Liatris* was most recently undertaken by Nesom (2005a) using morphological characters and geographic distributions, which split the 32 species across 2 sections with 11 series described by Gaiser (1946), into 37 species across 5 sections: *Liatris, Vorago, Graminifolium, Suprago, and Pilifilis* with 8 series. Sect. *Graminifolium* is comprised of ser. *Scariosae*, ser. *Gramnifoliae*, ser. *Virgate*, ser. *Pauciflorae*, and ser. *Garberae*. Sect. *Liatris* is comprised of: ser. *Liatris*, ser. *Punctatae*, ser. *Elgantes*. Polyploidy has occurred in ser. *Punctate* and possibly in other groups (Nesom, 2005a). Hybrids have been observed across infrageneric sections of this genus (Nesom, 2005a). The updated classification references Gaiser's (1946) classification scheme and credits her as the combining author of the series names, but provides proper Latin diagnoses for the groups. *L. helleri* is classified in the *Graminifoliae* subseries of *Graminifolium*. While these morphological features may be useful in some interspecific discrimination, a genetic approach using high-resolution markers is more appropriate in resolving species boundaries and areas of admixture in *Liatris* full of intraspecific variation and hybridization-potential.

Reproduction and Pollination

Species of *Liatris* are broadly sympatric, but ecologically distinct in their distribution, due to gradients of available nutrients, soil moisture, and elevation (Levin, 1967). Flowering in the genus occurs mostly in late summer through early fall. Some species may start flowering as early as June but all species bloom by August and sometimes continue into October (Weakley, 2015). Flowering in this genus allows periods of overlap in seasonal phenology even between the earliest and latest flowering species, which may facilitate hybridization in areas of sympatry (Levin, 1967). Species of

Liatris are predominately obligate outcrossers, displaying evidence of a genetic self-incompatibility mechanism (Levin, 1969; Godt & Hamrick, 1995). These species receive generalist pollination from a variety of insects and have wind-dispersed seeds. Pollination services in this genus are primarily provided by native bees of the *Bombus* genus, but also include varieties of butterflies and beetles. Bees and butterflies display high species specificity, while beetles do not discriminate between species (Levin, 1969; Godt & Hamrick, 1995). Levin (1969) observed flight distances of pollinators in *L. aspera* averaging 37 cm and seed dispersal distance averaging 2.49 m. He found a negative correlation between pollinator flight distance and pollen dispersal with plant density, leading him to conclude that pollinator flight distance and pollen dispersal are density-dependent. However, the main pollinator of this genus, bees, have been observed to fly distances of 8-11 km (5-7 mi) from their colony which also allows the possibility of gene flow between populations in relatively close proximity (Ribbands, 1951).

In cases of rare species such as *L. helleri*, small populations are thought to be less attractive to floral visitors, which may lead to pollinator scarcity (Agren, 1996). Wind-dispersal of seeds in small populations may also represent limitations in this genus. The lack of suitable habitats in adjacent areas is likely to limit seedling recruitment, and surrounding vegetation may capture and prevent dispersal of seeds, while strong winds in some habitats are likely to remove a majority of seeds from suitable habitats (Levin, 1969; Ulrey et al., 2016). Self-incompatibility in this genus may also limit the number of potential mates in small populations (Agren, 1996).

The combination of self-incompatibility, consistent chromosome morphology, generalist pollination regime, overlapping phenology, and wind-dispersion of seeds likely contribute to the sometimes blurry species boundaries in this genus. Any areas of relative sympatry of *Liatris* species have the potential to produce hybrid zones and complications in detecting species.

Genetic Markers

Plant biologists have long been interested in genetic methods of species delimitation. Some of the first attempts at this pursuit involved cytotaxomony, analyzing karyotypes to inform chromosome number and structure. One of the pioneers of this field was Lulu Odell Gaiser, who published many of the first organized lists of chromosome counts and is the taxonomic authority of *Liatris turgida* Gaiser (Funk, 2018). The advent of genetic markers has since allowed the field of plant biology to perform higher resolution investigations of species and population level questions by investigating genetic variation.

The first genetic markers were single trait morphological markers that were limited to species with phenotypic variation (e.g. flower color), one locus, and simple crosses (Cruzan, 1998). Codominant protein-based markers known as allozymes replaced this method. Allozyme markers utilize structural variation of enzymes to analyze variation of alleles at particular loci. These markers are not species specific, making them applicable across most study systems, but are usually evolutionary conserved due to their functional coding nature; thus they often fail to provide enough resolution for species level taxonomy. These markers proved their utility for decades in the field of evolutionary biology, ecology, and conservation biology by providing data comparable across studies and inheritance patterns closely associated with various life history traits (Nybom, 2004). Allozyme markers were used commonly throughout the 1970's into the 1990's but chemical exposure risks for researchers and the development of PCR (Polymerase Chain Reaction) led to these markers being replaced by more modern DNA-based markers.

PCR-based markers first utilized non-species specific primers, which allowed the investigation of wild plant species without prior sequence information. These markers included RAPDs (Random Amplified Polymorphic DNA) in 1990, ISSRs (Intersimple Sequence Repeats) in 1994, and AFLPs (Amplified Fragment Length Polymorphism) in 1995 (Nybom, 2004). The major drawback of these marker systems is that their reliance on non-specific primers and banding patterns that reveal presence/absence data for each locus. The inability to directly measure heterozygosity

means allele frequencies can only be approximated. The subsequent treatment of these markers systems as dominantly inherited has brought their application in estimating population genetic parameters such as diversity into question and debate (Nybom, 2004). Other drawbacks of these marker systems include poor reproducibility in RAPD markers and partial DNA digestion causing artifacts in AFLP markers. Methods such as RFLP (Restriction Fragment Length Polymorphism) provide an alternative as a co-dominant marker system with highly specific loci (NCBI, 2017). This marker system was utilized in phylogenetic and phylogeographic studies throughout the 1990s and is still in use today. This application of RFLP analyzes noncoding regions of cpDNA (chloroplast DNA) and has shown utility in large-scale phylogenies, but the slow evolutionary rates of cpDNA make investigating relationships within genera difficult without many loci (Bonatelli et al., 2013).

Microsatellites, or SSR (simple sequence repeats), were discovered in the 1980's and have been used in plant biology for over 20 years. Microsatellite markers are codominant markers composed of motifs of 1 to 10 nucleotides long, shorter than minisatellites (>10 nucleotides). Variation at these loci is thought to arise from polymerase strand-slippage during DNA replication or recombination errors (Viera et al., 2016). The high frequency of interspersed regions of repeats along with the remains from transposable elements makes these regions informative and relevant due to their fast rate of evolution. High mutation rates between 10^3 and 10^6 per cell generation make microsatellite regions highly polymorphic compared to coding regions (i.e., those detected with allozyme analysis) (Vieira et al., 2016). These variable repeat regions are found throughout the genome and are thought to be largely neutral, occurring mostly in non-coding regions. These characteristics of microsatellites make them ideal for measuring the genetic diversity and evolutionary potential in wild populations. However, here is increasing evidence that SSRs can originate in coding regions, leaving tandem repeats in many proteins that may contribute to the evolution of new proteins. Most SSRs in coding regions are tri- or hexa- nucleotide repeats that do not alter the reading frame. These tandem repeats are thought to facilitate evolution by providing ample variation, which could enable rapid development of new phenotypes (Vieira et al., 2016).

SSRs have proven their utility in population genetics, genome mapping, forensics, parentage analysis, and phylogeography. These loci can be fully amplified from poor quality or low quantity DNA samples, lending to their application in ancient DNA or herbarium specimens (Hodel et al., 2016). The main drawback of SSRs was the time and cost of development of species-specific primers from genomic libraries (Nybom, 2004). However, advances in Next Generation Sequencing (NGS) technology has allowed genomic libraries to be built with relative ease and low cost (see Chapter 2). These libraries are then mined for microsatellite sequences using programs like MSATCommander (Faircloth, 2008) to develop species-specific primers for these loci. The developed primers are then screened for polymorphism and transferability in closely related species (Vieira et al., 2016). In the near future, SNPs (single nucleotide polymorphism), a biallelic marker system, will likely take the place of microsatellites once the cost of sequencing reference genomes becomes more affordable. These makers look at single nucleotide polymorphisms at thousands of loci, allowing the construction of enormous datasets and offering high resolution. SNPs have already been employed in several systems, including among Galapagos tortoises to identify distinct genetic groups and admixture in 12 genetic lineages (Miller et al., 2018).

Microsatellites are not without drawbacks. These markers are prone to back mutations that could lead to homoplasy, complicating analyses of genotype data. Their high rates of polymorphism also make them less than ideal for genetic analyses beyond closely related taxa. Genotyping errors can lead to downstream bias and high allele frequencies per locus can lead to inflated F-statistics (Hodel et al., 2016). For situations involving rare species or small budgets (e.g. conservation genetics), however, microsatellites remain the most cost effective and efficient choice for population genetic studies (Vieira et al., 2016; Hodel et al., 2016).

Genetic Variation

Rare species are typically characterized by restricted geographic ranges and small populations. A review of 57 genera found reduced rates of polymorphism, number of alleles, and

heterozygosity in 75% of rare species compared to their common congeners (Cole, 2003). Population genetic theory proposes that small populations lose genetic variation quickly and that this loss of variation can be catalyzed by genetic drift, reduction in gene flow, and inbreeding (Ellstrand and Elam, 1993). The reduction of genetic diversity through these processes can reduce population fitness and decrease the evolutionary potential of taxa (Ellstrand and Elam, 1993; Reed and Frankham, 2003). This effect could potentially leave rare species even more vulnerable to extinction in the face of threats such as climate change and habitat degradation, loss, and/or fragmentation. However, there are various scenarios influenced by life history traits, spatial distribution, and evolutionary history through which rare species can display high levels of genetic variation. It is critical to understand the genetic dynamics of these species in order make the most informed management decisions.

Rarity in plant species is not always accompanied by reduced levels of genetic variation and increased levels of genetic structure. Common species are typically characterized by a wide geographic range, broad habitat specificity, large populations, and high genetic diversity (Hamrick & Godt, 1996; Rabinowitz, 1981). However, common species do not display significant differences in genetic structure or gene flow when compared to rare species (Cole, 2003). In studies of *Acer saccharum* (Sugar Maple), populations are known to exhibit higher gene flow than larger, more connected populations over a similar range (Foré et al., 1992) Rare species have a variety of spatial and temporal characters that do not always result in the typical genetic signature of highly structured populations with low diversity (Binks et al., 2015), although there are always exceptions to the norm. Attempts at classifying various types of rarity should take into account a variety of factors that may influence genetic diversity such as biogeographic history, geographic distribution, breeding system, and life history traits (Hamrick and Godt, 1996; Rabinowitz, 1981; Rossetto et al., 2008). Rossetto et al. (2008) offered three explanations of rarity based on evolutionary history: (1) early stages of expansion, (2) nearing the end of a species' decline, (3) persistence of a natural rarity throughout time. *Liatris helleri* likely exhibits a type of rarity that has been shaped through its persistence within a natural community throughout time and anthropogenically induced population declines that have

led to its current state. Since the high elevation rock outcrop community that this species inhabits is thought to represent a floral community of post-Pleistocene alpine relic species, *L. helleri* likely represents a fellow member of this community (Russell et al., 2009). The persistence of these communities throughout time could also offer an explanation for the high-observed genetic diversity of *L. helleri* (Godt & Hamrick, 1996).

Species Boundaries and Previous Studies

Species exist as clusters of genetic and ecological similarity, and the act of speciation is driven mainly by natural selection (Shapiro et al., 2016). Ernst Mayr contributed greatly to our current day conceptualization of species, built on the fundamental idea of individually evolving metapopulation lineages through his conceptualization of the Biologicial Species Concept, from which a plethora of species concepts have emerged. The framing of the definition of a species is critical to systematics and conservation, but there is no universal consensus on the degree of divergence that separates lineages into separate species (de Queiroz, 2007). The ecological, evolutionary, phylogenetic, and genotypic cluster species concepts are all relevant to this research. The ecological species concept separates species based on organisms living in the same niche or adaptive zone such as high elevation rock outcrops or shale barrens (Andersson, 1990). This species concept allows connection between the environment and patterns of variation, but has been criticized in its inability to define species due to limits in specifying a species niche. Instead, this concept should be used a secondary criteria in taxonomic delineations (Andersson, 1990). The evolutionary species concept was developed by Simpson (1951) to address issues with the Biological Species Concept by accounting for extinct species and asexual organisms. It diagnoses species based on their unique evolutionary role and tendencies along with their historical fates. However, the utility of this concept has encountered limitations when tasked with explaining gaps in the fossil record that levy prejudice limits between species (Aldhebiani, 2018). The phylogenetic species concept umbrella can be divided into character-based concepts, which define a species as the smallest aggregate of

populations or lineages that is diagnosable by a unique combination of character states, and history based approaches, which define a species based on the coalescence of alleles from a common ancestor (Baum & Shaw, 1995; Baum & Donoghue, 1995). These methods have been criticized for their relative inability to reconstruct evolutionary pathways with certainty and possible overestimation of intraspecific diversity through taxonomic inflation (Aldhebiani, 2018; Stanton et al., 2019). The genotypic cluster concept relies on identifying unique genetic clusters, those with few heterozygous or genetically intermediate individuals (Mallet, 1995). This method can be useful in identifying distinct genetic groupings of an organism, but lacks clearly defined limits on what separates taxa and is therefore not useful in defining taxonomic delineations. This could, however, be useful as secondary criteria in conceptualizing a species or its population structure.

The species concept most applicable to this system is the unified species concept proposed by de Quieroz (2007). This concept seeks to create a universally compatible species concept that treats all species as segments of separately evolving groups of regionally connected populations or metapopulation lineages and all other conceptual frameworks as secondary properties that describe different points along a speciation gradient. This concept separates the conceptualization of species from the methodical problem of inferring boundaries between species (de Quieroz, 2007). Under the unified species concept all lines of evidence are relevant in the delineation of species, which creates a synergistic approach in the delineation of species boundaries. Any disagreements between species delineation under this concept should arise from relevance of data, temporal scale, prospective versus retrospective evidence, and cases of incomplete lineage separation (de Quieroz, 2007).

There are a growing number of studies utilizing this multivariate approach to defining species. A combination of mitochondrial markers, climate and elevation data, and morphological data was used to successfully delineate species boundaries in the *Oophaga* genus of neotropical frogs to inform conservation strategies and taxonomic uncertainties (Posso-Terranova and Andres, 2018). Geographical, morphological, and mitochondrial genetic data were used in combination to define 40 species boundaries in avian genera of the Philippine archipelago (Hosner et al., 2018). The

interdisciplinary approaches displayed in these studies highlight the importance of collaborative science to achieve the most comprehensive understanding of a species. Properties of any alternative species concept that lend evidence of lineage separation are relevant to defining species boundaries and number of species. A single property of speciation does not guarantee separate lineages but acts as evidence and complete separation of lineages is only appropriate with multiple lines of evidence. Only an absence of all properties should be considered as evidence against separate species distinctions (de Quieroz, 2007).

Previous studies of *L. helleri* offer some framework to build a multivariate argument of speciation. Godt and Hamrick (1996) observed high genetic diversity in allozyme markers, found low levels of gene flow, and inferred evidence of population structure via isolation. Nesom (2005b) found no combination of morphological characters that could separate *L. helleri* and *L. turgida*. This study also cited the high genetic diversity observed by Godt and Hamrick (1996) as further evidence of a more widespread, interbreeding taxon. Sullins (2013) found incomplete lineage separation between the two taxa using cpDNA markers. The only findings from these studies that could contribute as evidence toward speciation in these taxa was the low level of gene flow observed in *L. helleri* (Godt & Hamrick, 1996), but these authors did not look at *L. helleri* in relation to *L. turgida*. The lack of morphological differentiation and incomplete lineage sorting with cpDNA found in these studies offer evidence against separate lineages in these taxa. An analysis of ecological data for their respective habitats such as soil composition, elevation, aspect, temperature, and precipitation would be appropriate in this system to elucidate plausible species boundaries between these taxa.

Liatris helleri Porter (Asteraceae), Heller's blazing star, is a rare perennial herb endemic to the high elevation rock outcroppings of the Southern Appalachians (Figure 1). This species receives generalist pollination from a variety of bees, butterflies, and moths and exhibits a restricted geographic range comprised of less than 8 extant populations throughout western North Carolina, all within a 30 km radius of each other (Godt & Hamrick, 1995; Murdock, 2000). The Shale Barren Blazing Star, *L. turgida* Gaiser, is a lower elevation species with similar morphology that occurs

throughout shale barrens of central Appalachia. The separation of these two taxa as individual species has been a point of contention due to previous studies of genetic diversity and morphology, suggesting the possibility that together they may represent a single regionally distributed species.

Figure 1. *Liatris helleri* in a high elevation rock outcrop community in Avery County, NC.

The high genetic diversity observed in *L. helleri* may be an example of such exceptions to the typical assumptions of rare species. *L. helleri* exhibits a restricted geographic distribution, typically small population sizes, self-incompatibility, and wind-dispersion of seeds, and high habitat specificity, which would classify it as a restricted endemic under Rabinowitz's (1981) treatment. Self-incompatibility in this species represents a limitation of breeding in small populations, which typically contain closely related individuals and are less conspicuous to pollinators (Goodell et al,. 1997). Even if individuals were able to achieve successful seed set with a closely related neighbor in

their population, this scenario would likely lead to inbreeding and reduced genetic variation. Low levels of gene flow and evidence of biparental inbreeding have been observed in *L. helleri* and likely contribute to the population differentiation, but high levels of genetic diversity are somewhat confounding (Hamrick and Godt, 1996).

This species may be a relic species restricted to glacial refugia from past climatic cycles, like other species found in high elevation species such as *Geum radiatum* (Spreading Avens) and *Solidago spithamaea* (Blue Ridge Goldenrod) (Ulrey et al., 2016; Wiser et al., 1996). In this scenario the persistence of this species throughout time in a specialized niche could account for the higher than expected levels of observed diversity (Rosetto, 2008; Binks et al., 2015). Another plausible scenario to consider is that *L. helleri* is actually successfully outcrossing across a larger geographic range, facilitated by wind-dispersed seeds and self-incompatibility. As noted earlier, *Liatris* species are known to form hybrids when they occur in sympatry (Levin, 1968). Areas such as Shortoff Mt. in the southern end and Bluff Mt. at the northern end of *L. helleri*'s range are known to harbor multiple species of *Liatris* (NCNHP, 2019). These areas have the potential to act as stepping-stones of outcrossing along the Appalachian mountain range from the high elevation rock outcrops of southern Appalachia to the shale barrens of central Appalachia. The increased opportunity for outcrossing in areas like these could provide an explanation for the high genetic diversity observed in Hamrick and Godt (1996).

The Godt and Hamrick (1996) study falls short in that it does not knowingly compare populations of *L. helleri* to its congener *L. turgida*. Populations in the Linville Gorge and on Grandfather Mountain that were identified as admixed or predominately *L. turgida* by the current study were a part of their sampling and may have led to erroneous interpretation of results. Previous attempts employing cpDNA markers (Sullins, 2013) provided too coarse of resolution to delineate clear boundaries between the taxa, due to the highly conserved nature of the markers. This study concluded that *L. helleri* and *L. turgida* were experiencing incomplete lineage sorting or effectively in the midst of a speciation event. An investigation of these species using high-resolution genetic

markers (e.g. microsatellites) to evaluate gene flow, differentiation, and genetic structure throughout the range of both of these species should help resolve taxonomic uncertainties in this system. It is possible that there are not easily diagnosable characters for this species. Areas of admixture are a distinct possibility, even if the two taxa are genetically divergent, and may complicate taxonomic classification. The implementation of a microsatellite analysis in this system should provide the ability to identify possible hybrid zones along with genetically distinct populations of both taxa. The data generated from this method will likely prove helpful in conservation management strategies along with providing high-resolution genetic data in the taxonomic delineation of these taxa.

Recreation and Fire Suppression

Liatris helleri shares its home with some of the most scenic and sought after views in the High Country. Rock climbers, high-liners, tourists, and hikers all seek to experience the natural beauty found in these high elevation rock outcrop communities, but not without a price. The most immediate threat posed to this species and its fellow community inhabitants is that of trampling (Murdock, 2000). During the 2018 field season, several trampled individuals were observed among the more popular localities. Recreational activities such as rock climbing and high-lining also occurred with no regard for the sensitivity of the surrounding plant communities. Significant population declines correlated to recreational intensity have been observed in this species, even after the installation of a boardwalk to direct hikers away to avoid trampling (Sutter et al., 1993). Increased educational outreach, signage, permitting for recreational activities, trail barriers, and limiting access to certain populations will likely be crucial steps in preserving this species and its natural habitat.

L. helleri and other members of the genus *Liatris* are known to exhibit positive responses to prescribed fire management and have displayed t growth increases of up to 157% after two burns (Medve, 1987). Some high elevation rock outcrops are subject to natural succession and experience shading due to shrubby vegetation accumulation that typically climaxes in spruce-fir forests at these elevations (Murdock, 2000). Several populations of *L. helleri* have been burned in the past and have

shown positive results of increased recruitment (C. Ulrey, per. comm.). Fire maintenance in these communities would likely provide more habitat for open light species such as *L. helleri*. One of the Element Occurrences (EOs) visited during the 2018 field season had been subjected to wildfire and displayed the largest number of flowering individuals visited during my study. The establishment of a fire regime for these populations would likely prove a useful management tool in the preservation of this species.

Climate Change

The rapid changes occurring in our environment pose threats to all species by compromising ecosystem productivity, disrupting species interactions, and facilitating the spread of invasive species. Rare species or those with restricted ranges may be even more vulnerable to climate change when faced with the compounding effects of increased habitat fragmentation and degradation, leaving these species with less range connectivity and reduced adaptive abilities that increase their likelihood of extinction (USGCRP, 2018; Pitelka, 1997).

High elevation species are at an elevated risk of extinction due to climate change (Dirnbock et al., 2011). The microclimatic environments in these habitats provide a buffer from macroclimatic conditions, but elevated temperatures compounded by stochastic environmental events and loss of suitable habitat threatens to disturb the maintenance of these small and restricted natural communities. A projected 53-85% loss of suitable habitat by the year 2080 is predicted for *Geum radiatum*, a fellow endemic of high elevation rock outcrops in the southern Appalachians (Ulrey et al., 2016). The intensity of current climate change is likely to impact a variety of endemic and rare species that are range-restricted within specialized habitats. The current range restrictions of these rare high-elevation species may reflect poor dispersal abilities and retention traits selected for during environmental conditions of the last ice age (Dirnbock et al., 2011).

Another possible concern in the preservation of *L. helleri* is the loss of pollinator services. Wells and Tonkyn (2018) projected 91% declines in suitable habitat by 2050 for *Speyeria diana*, a

southern Appalachian butterfly known to inhabit high elevation habitats and which already has a restricted range. *L. helleri* is known to receive pollination from a variety of species of bees, butterflies, and moths; this might benefit this species in a future environment with limited pollinators. This species also exhibits self-incompatibility and wind dispersal of seeds, but usually occurs in populations of small numbers ranging from ten to several hundred flowering plants (Godt & Hamrick, 1995; Clark, personal observation). These smaller populations are thought to be less attractive to floral visitors, which might further contribute to pollinator scarcity in this species (Agren, 1996).

It is clear that climate change poses threats across ecosystems globally, but rare species with already limited ranges and specific habitat requirements are likely to be affected at disproportionate rates (Dirnbock et al., 2011). *L. helleri* is no exception to this reality. Small isolated populations increase its susceptibility to extinction events and its restriction to cool mountain top habitats poses an additional limit to its migration possibilities across a fragmented landscape. Management initiatives will require proactive measures to alleviate these threats in the hope of persevering this species.

References:

Aldhebiani, A. Y. (2018). Species concept and speciation. *Saudi Journal of Biological Sciences*, *25*, 437–440. https://doi.org/10.1016/j.sjbs.2017.04.013

Allen-Wardell, G. (2008). The Potential Consequences of Pollinator Declines on the Conservation of Biodiversity and Stability of Food Crop Yields. *Conservation Biology*, *12*, 8–17. https://doi.org/10.1111/j.1523-1739.1998.97154.x

Agren, J. (1996). Population Size, Pollinator Limitation, and Seed Set in the Self- Incompatible Herb *Lythrum Salicaria*. *Ecology*, *77*, 1779–1790. https://doi.org/10.2307/2265783

Andersson, L. (1990). The Driving Force: Species Concepts and Ecology. *Taxon*, *39*, 375. https://doi.org/10.2307/1223084

Baum, D., & Shaw, K. (1995). Genealogical Perspective on the Species Problem. *Experimental and Molecular Approaches to Plant Biosystematics*, *53*, 289–303.

Baum, D. A., & Donoghue, M. J. (1995). Choosing among Alternative "Phylogenetic" Species Concepts. *Systematic Botany*, *20*, 560. https://doi.org/10.2307/2419810

Binks, R. M., Millar, M. A., & Byrne, M. (2015). Not all rare species are the same: contrasting patterns of genetic diversity and population structure in two narrow-range endemic sedges: Comparative Genetics of Rare *Lepidosperma*. *Biological Journal of the Linnean Society*, *114*, 873–886. https://doi.org/10.1111/bij.12465

Bonatelli, I. A. S., Zappi, D. C., Taylor, N. P., & Moraes, E. M. (2013). Usefulness of cpDNA markers for phylogenetic and phylogeographic analyses of closely related cactus species. *Genetics and Molecular Research*, *12*, 4579–4585. https://doi.org/10.4238/2013.February.28.27

Cole, C. T. (2003). Genetic Variation in Rare and Common Plants. *Annual Review of Ecology, Evolution, and Systematics*, *34*, 213–237. https://doi.org/10.1146/annurev.ecolsys.34.030102.151717

Cruzan, M. B. (1998). Genetic Markers in Plant Evolutionary Ecology. *Ecology*, *79*, 400. https://doi.org/10.2307/176941

de Queiroz, K. (2007). Species Concepts and Species Delimitation. *Systematic Biology*, *56*, 879–886. https://doi.org/10.1080/10635150701701083

Dirnböck, T., Essl, F., & Rabitsch, W. (2011). Disproportional risk for habitat loss of high-altitude endemic species under climate change: Habitat Loss of High Altitude Endemics. *Global Change Biology*, *17*, 990–996. https://doi.org/10.1111/j.1365-2486.2010.02266.x

Ellstrand, N. C., & Elam, D. R. (1993). Population Genetic Consequences of Small Population Size: Implications for Plant Conservation. *Annual Review of Ecology and Systematics*, *24*, 217–242. https://doi.org/10.1146/annurev.es.24.110193.001245

Faircloth, B. C. (2008). msatcommander: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources*, *8*, 92–94. https://doi.org/10.1111/j.1471-8286.2007.01884.x

Foré, S. A., Hickey, R. J., Vankat, J. L., Guttman, S. I., & Schaefer, R. L. (1992). Genetic structure after forest fragmentation: a landscape ecology perspective on *Acer saccharum*. *Canadian Journal of Botany*, *70*, 1659–1668. https://doi.org/10.1139/b92-205

Funk, V. (2018). Unsung heroes of botany: Lulu Odell Gaiser. *Taxon*, *67*, 1053–1054. https://doi.org/10.12705/675.28

Gaiser, L. O. (1946). The Genus *Liatris*. *Rhodroa*, *48*, 331–382.

Gaiser, L. O. (1949). Chromosome Studies in *Liatris*. I. Spicatae and Pycnostachyae. *American Journal of Botany*, *36*, 122. https://doi.org/10.2307/2438129

Gaiser, L. O. (1950). Chromosome Numbers in *Liatris*. II. Graminifoliae and Pauciflorae. *American Journal of Botany*, *37*, 414. https://doi.org/10.2307/2438213

Godt, M. J. W., & Hamrick, J. L. (1995). The mating system of *Liatris helleri* (Asteraceae), a threatened plant species. *Heredity*, *75*, 398–404. https://doi.org/10.1038/hdy.1995.152

Godt, M. J. W., & Hamrick, J. L. (1996). Genetic diversity and morphological differentiation in *Liatris helleri* (Asteraceae), a threatened plant species. *Biodiversity and Conservation*, *5*, 461–471. https://doi.org/10.1007/BF00056391

Goodell, K., Elam, D. R., Nason, J. D., & Ellstrand, N. C. (1997). Gene flow among small populations of a self-incompatible plant: an interaction between demography and genetics. *American Journal of Botany*, *84*, 1362–1371. https://doi.org/10.2307/2446134

Hamrick, J. L., & Godt, M. J. W. (1996). Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *351*, 1291–1298. https://doi.org/10.1098/rstb.1996.0112

Hodel, R. G. J., Segovia-Salcedo, M. C., Landis, J. B., Crowl, A. A., Sun, M., Liu, X., … Soltis, P. S. (2016). The Report of My Death was an Exaggeration: A Review for Researchers Using Microsatellites in the 21st Century. *Applications in Plant Sciences*, *4*, 1600025. https://doi.org/10.3732/apps.1600025

Hosner, P. A., Campillo, L. C., Andersen, M. J., Sánchez-González, L. A., Oliveros, C. H., Urriza, R. C., & Moyle, R. G. (2018). An integrative species delimitation approach reveals fine-scale endemism and substantial unrecognized avian diversity in the Philippine Archipelago. *Conservation Genetics*, *19*, 1153–1168. https://doi.org/10.1007/s10592-018-1085-4

Levin, D. A. (1967). An Analysis of Hybridization in *Liatris*. *Brittonia*, *19*, 248. https://doi.org/10.2307/2805776

Levin, D. A. (1968). THE STRUCTURE OF A POLYSPECIES HYBRID SWARM IN *LIATRIS*. *Evolution*, *22*(2), 352–372. https://doi.org/10.1111/j.1558-5646.1968.tb05903.x

Levin, D. A. (1969). CHROMOSOME PAIRING IN NATURAL FIRST AND ADVANCED GENERATION LIATRIS HYBRIDS. *American Journal of Botany*, *56*, 592–599. https://doi.org/10.1002/j.1537-2197.1969.tb07574.x

Mallet, J. (1995). A species defnition for the Modern Synthesis. *Tree*, *10*.

Medve, R. J. (1987). The Effects of Fire on Resource Allocation and Growth of *Liatris spicata*. *American Midland Naturalist*, *117*, 199. https://doi.org/10.2307/2425720

Miller, J. M., Quinzin, M. C., Edwards, D. L., Eaton, D. A. R., Jensen, E. L., Russello, M. A., … Caccone, A. (2018). Genome-Wide Assessment of Diversity and Divergence Among Extant Galapagos Giant Tortoise Species. *Journal of Heredity*, *109*, 611–619. https://doi.org/10.1093/jhered/esy031

Murdock, N (2000). Recovery Plant for *Liatris helleri*, Heller's Blazing Star. US Fish and Wildlife Service.

Nesom, G. (2005a). Infrageneric Classificaiton of *Liatris* (Asteraceae: Eupatorieae). *SIDA, Contirbutions to Botany*, *21*, 1305–1321.

Nesom, G. (2005b). Broadened Concept of *Liatris helleri* (Asteraceae: Eupatorieae). *SIDA*, *21*, 1323–1333.

North Carolina Natural Heritage (NCNHP) (2019) Element Occurence Report for *Liatris helleri.*

Nybom, H. (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants: DNA -Markers and Plant Genetic Diversity. *Molecular Ecology*, *13*, 1143–1155. https://doi.org/10.1111/j.1365-294X.2004.02141.x

Pitelka, L., & Plant Migration Workshop Group. (1997). Plant migration and climate change. *American Scientist*, *85*, 464–473.

Posso-Terranova, A., & Andrés, J. (2018). Multivariate species boundaries and conservation of harlequin poison frogs. *Molecular Ecology*, *27*, 3432–3451. https://doi.org/10.1111/mec.14803

Rabinowitz, D. (1981). Seven forms of rarity. *The Biological Aspects of Rare Plant Conservation*.

Reed, D. H., & Frankham, R. (2003). Correlation between Fitness and Genetic Diversity. *Conservation Biology*, *17*, 230–237. https://doi.org/10.1046/j.1523-1739.2003.01236.x

National Center for Biotechnology Information (NCBI). (2017). Restriction Fragment Length Polymorphism (RFLP). NCBI. Retrieved from https://www.ncbi.nlm.nih.gov/probe/docs/techrflp/

Ribbands, C. R. (1951). The Flight Range of the Honey-Bee. *The Journal of Animal Ecology*, *20*, 220. https://doi.org/10.2307/1541

Rossetto, M., Kooyman, R., Sherwin, W., & Jones, R. (2008). Dispersal limitations, rather than bottlenecks or habitat specificity, can restrict the distribution of rare and endemic rainforest trees. *Americna Journal of Botany*, *95*, 321–329.

Russell, D. A., Rich, F. J., Schneider, V., & Lynch-Stieglitz, J. (2009). A warm thermal enclave in the Late Pleistocene of the South-eastern United States. *Biological Reviews*, *84*, 173–202. https://doi.org/10.1111/j.1469-185X.2008.00069.x

Shapiro, B. J., Leducq, J.-B., & Mallet, J. (2016). What Is Speciation? *PLOS Genetics*, *12*, e1005860. https://doi.org/10.1371/journal.pgen.1005860

Simpson, GG. (1951). The Species Concept. *Evolution*, *5*, 285–298.

Stanton, D. W. G., Frandsen, P., Waples, R. K., Heller, R., Russo, I.-R. M., Orozco-terWengel, P. A., Bruford, M. W. (2019). More grist for the mill? Species delimitation in the genomic era and its

implications for conservation. *Conservation Genetics*, *20*(1), 101–113. https://doi.org/10.1007/s10592-019-01149-5

Sullins, P. (2013). USING cpDNA to Assess Species Delimitation Between *Liatris helleri* Porter (Asteraceae) and *Liatris turgida* Gaiser. Appalachian State University.

Sutter, R.D., Benjamin, S.E., Murdock, M., Teague, B. (1993) Monitoring the Effectiveness of a Boardwalk at Protecting a Low Heath Bald in the Southern Appalachians. *Natural Areas Journal* 13: 250-255

Ulrey, C., Quintana-Ascencio, P. F., Kauffman, G., Smith, A. B., & Menges, E. S. (2016). Life at the top: Long-term demography, microclimatic refugia, and responses to climate change for a high-elevation southern Appalachian endemic plant. *Biological Conservation, 200*, 80–92. https://doi.org/10.1016/j.biocon.2016.05.028

US Global Change Research Program (USGCRP). Fourth National Climate Assessment; Volume II: Impacts, Risks, and Adaptation in the United States.

Vieira, M. L. C., Santini, L., Diniz, A. L., & Munhoz, C. de F. (2016). Microsatellite markers: what they mean and why they are so useful. *Genetics and Molecular Biology*, *39*, 312–328. https://doi.org/10.1590/1678-4685-GMB-2016-0027

Wiley, EO. (1981). Remarks on Willis' Species Concept. *Systematic Zoology*, *30*, 86–87.

Wiser, S., Peet, R., & Peter, W. (1996). High-elevation rock outcrop vegetation of the Southern Appalachian Mountains. *Journal of Vegetation Science, 7*, 703–722.

Weakley, A. (2015). *Flora of the Southern and Mid-Atlantic States*. UNC Herbarium, North Carolina Botanical Garden, University of North Carolina at Chapel Hill.

Wells, C., & Tonkyn, D. (2018). Changes in the Geographic Distribution of the Diana Fritillary (*Speyeria diana*: Nymphalidae) under Forecasted Predictions of Climate Change. *Insects*, *9*, 94. https://doi.org/10.3390/insects9030094

Chapter 2

Development of Seventeen Microsatellite Markers in the Federally Endangered, *Liatris helleri* (Asteraceae)

Logan C. Clark, Morgan R. Gaglianese-Woody, Matt C. Estep

Department of Biology, Appalachian State University, 572 Rivers Street, Boone NC, 28607-2027 USA

Email addresses: LCC: clarklc@appstate.edu

MRGW: gaglianesewoodymr@appstate.edu

MCE: estepmc@appstate.edu

Number of words: ~1126

Manuscript received_____; revision accepted_____

Author for correspondence: estepmc@appstate.edu

ACKNOWLEDGEMENTS

The authors would like to thank the Conservation Genetic class of 2016 for their contribution to this project and G. Kauffman (USFS) for his assistance in the collection of these samples. Funding provided by USFW (F16AP00773). Permits provided by NC-PCP, NPS, USFS, USFWS.

ABSTRACT

- *Premise of the study*: Microsatellite markers were developed in the federally endangered, *Liatris helleri* (Asteraceae), to evaluate species boundaries with closely related congeners within the genus.
- *Methods and Results*: Using Illumina data, 17 primer pairs were developed in populations of *L. helleri*. The primers amplified motifs from tri- to hexanucleotide repeats with 1-17 alleles per locus. Primers were also tested for cross-amplification in *L. aspera, L. microcephela,* and *L. pycnostachya*.
- *Conclusions:* The developed primers for *L. helleri* serve as a novel genetic tool for future investigations in this genus, allowing for more explicit species delineation as well as population genetic analyses.

Key Words: Asteraceae; endangered species; *Liatris helleri*; perennial herb; southern Appalachians; species boundaries.

INTRODUCTION

The North American genus *Liatris* (Asteraceae, Asterales) is composed of 40-50 species, mainly confined to the eastern seaboard of North America (Gaiser, 1946; Weakley, 2015). *Liatris* has been considered a genus of "unusual difficulty" due to variability and hybridization between species that has led to unclear delineation of species boundaries (Gaiser, 1946). Species of *Liatris* are broadly sympatric, but ecologically distinct in their distribution, which is related to gradients of available nutrients, soil moisture, and elevation (Levin, 1967). Phenology in the genus occurs mostly in late summer through early fall, but periods of overlap in seasonal phenology even between the earliest and latest flowering species may facilitate hybridization in areas of sympatry (Levin, 1967). Morphological distinctions in this genus are not abundant and have led to somewhat blurry species delineations. This has been the case with *Liatris helleri* Porter and its closely related congener, *L. turgida* Gaiser (Gaiser, 1946; Nesom, 2005b). Since *L. helleri* is listed as federally endangered, it is

crucial for land managers and conservationists alike to have a clear concept of the boundaries of this species.

METHODS AND RESULTS

DNA was extracted from a single individual of *L. helleri* (Appendix 1) using a modified CTAB method (Doyle and Doyle, 1987). An Illumina MiSeq sequencing library was constructed and paired-end sequenced at the West Virginia Core facility. Raw sequence reads were quality controlled and trimmed using fastp (Chen et al., 2018). A total of 18,020,464 sequence reads were queried by MSATCOMMANDER version 1.0.8 (Faircloth, 2008) with default settings, minimum primer size was set at 20 bp, maximum primer GC content was limited to 50%, and a PIG-tail sequence (GTTT) (Brownstein et al., 1996) was added to one primer. A total of 192,645 microsatellite loci were identified, 6,919 of which were suitable for primer design.

Three populations, each composed of multiple subpopulations, were sampled by collecting single leaf samples from individuals (Appendix 1). Samples were then stored on silica gel and placed in a -80°C freezer until used for DNA extraction. Extractions were performed using the PureLink Plant Total DNA Purification Kit (Invitrogen, Carlsbad, CA, USA). One hundred and nineteen primers pairs were tested by amplifying under standard conditions in a small group of individuals. PCR reactions were prepared in a 10-µL volumes consisting of 1× Go Taq Flexi Buffer, 2.5 mM $MgCl_2$, 800 µM dNTPs, 0.5 µM each primer, 0.5 units Go Taq Flexi DNA Polymerase (Promega Corporation, Madison, Wisconsin, USA), and 1 µL of DNA. PCR was completed using a touchdown thermal cycling program on an Eppendorf Mastercycler (Eppendorf, Hauppauge, New York, USA) with annealing temperatures ranging from 68°C to 55°C. Initial denaturation was 94°C for 5 min, followed by 13 cycles (45 s at 94°C, 2 min at annealing temperature, and 1 min at 72°C), followed by 24 cycles (45 s at 94°C, 1 min at 55°C, and 1 min at 72°C), followed by 10 min at 72°C.

PCR products were examined on a 1% agarose gel and scored for the presence or absence of an appropriately sized PCR product and uniform amplification across samples. A total of 20 primers consistently amplified and were further examined by pseudo-multiplexing fluorescently labeled PCR

products with 6-FAM, VIC, NED, or PET by adding 0.25 μM of an M13 primer (5'-CACGACGTTGTAAAACGAC-3') to the PCR reaction following Schuelke (2000). PCR products were pooled and combined with a GeneScan 500 LIZ Size Standard (Life Technologies, Carlsbad, California, USA) for genotyping on an ABI 3730xl DNA Analyzer at the Georgia Genomics Facility (Athens, Georgia, USA). Resulting chromatograms were scored using Geneious 9.1.5 (Kearse et al., 2012; Biomatters Ltd., Auckland, New Zealand). Markers displaying more than two alleles for a single individual or failing to be easily scorable were removed from further analysis. The resulting genotypic data were analyzed using GenAlEx version 6.503 (Peakall and Smouse, 2012) to obtain standard descriptive statistics and test for per population deviations of HWE at each locus. The presence of null alleles was tested using Microchecker (Van Oosterhoutet al., 2004). Tests for linkage disequilibrium (LD) and global exact tests of heterozygosity deficiency were performed in GENEPOP using default Markov chain parameters (Rousset 2008).

Seventeen of the primer pairs consistently amplified and produced chromatograms that were easily scored. Three of these markers (LH2, LH4, and LH24) were monomorphic (Table 1). The remaining fourteen polymorphic markers produced from 2 to 17 alleles per locus with an average of 6.0 (Table 2). The effective number of alleles per locus ranged from 1.09 to 10.00 with an average of 3.38 (Table 2). Expected heterozygosites ranged from 0.135-0.900 with an average of 0.640 (Table 2). Markers LH14, LH21, LH22, LH68, and LH78 showed evidence for the presence of null alleles. Observed heterozygosities tended to be lower than expected, which aligns with results from a previous study using allozyme markers (Godt and Hamrick, 1996). The excess of homozygotes indicated by a global exact test ($P<0.000$) were not consistent across populations and could also be due to the Wahuland effect caused by sampling very small sub-populations of this federally listed species (Table 2). Significant LD was detected between marker pair LH22/LH83 ($P<0.001$) and marker pairs LH10/LH21, LH10/LH22, LH25/LH67, and LH16/LH69 ($P<0.05$).

Cross amplification experiments were performed by extracting DNA from 5 individuals from each of three species: *L. aspera* Michx., *L. microcephela* (Small) K. Schum., and *L. pycnostachya*

Michx. (Table 3 & Appendix 1). Each species was chosen to represent a different clade within the genus (Nesom, 2005a).

CONCLUSIONS

The seventeen microsatellite markers developed here will be a useful tool to investigate the genetic diversity of *L. helleri* species and can be used to better understand species boundaries between *L. helleri* and *L. turgida*. These markers also displayed the ability to cross amplify in *L. aspera*, *L. microcephela*, and *L. pycnostachya*, each representing distinct clades within the genus, suggesting these markers will provide the ability to assess genetic diversity of these species. The application of these markers should lead to a more thorough understanding of the dynamic properties of this genus while providing data for more efficient management and conservation strategies.

LITERATURE CITED

Brownstein, M.J., J. D. Carpten, and J.R. Smith. 1996. Modulation of Non-Templated Nucleotide Addition by *Taq* DNA Polymerase: Primer Modifications that Facilitate Genotyping. *BioTechniques* 20: 1004–1010.

Chen, S., Z. Yanqing, C. Yaru, and G. Jia. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor, *Bioinformatics* 31(17): 884–890

Doyle, J.J., and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11 - 15

Faircloth, B. C. 2008. msatcommander: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* 8: 92–94.

Gaiser, L. O. 1946. The Genus Liatris. *Rhodroa* 48(575): 331–382.

Godt, M. J. W., and J.L. Hamrick. 1996. Genetic diversity and morphological differentiation in Liatris helleri (Asteraceae), a threatened plant species. *Biodiversity and Conservation* 5: 461–471.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Drummond, A. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649.

Levin, D. A. 1967. An Analysis of Hybridization in *Liatris*. *Brittonia* 19(3): 248.

Nesom, G. 2005a. Infrageneric Classification of *Liatris* (Asteraceae: Eupatorieae). *SIDA, Contributions to Botany* 21: 1305–1321.

Nesom, G. 2005b. Broadened Concept of *Liatris helleri* (ASTERACEAE: EUPATORIEAE). *Contributions to Botany* 21(3): 1323–1333.

Peakall, R. and P.E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. *Bioinformatics* 2: 2537–2539.

Rousset, F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Mol. Ecol. Resources* 8: 103-106.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

Van Oosterhout, C., W.F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.

Weakley, A. 2015. Flora of the Southern and Mid-Atlantic States. University of North Carolina Herbarium, Chapel Hill, North Carolina, USA

TABLES

Table 1. Characteristics of 17 microsatellite markers developed for *Liatris helleri*.

Locus	Primer Sequence (5'-3')	Repeat Motif	Allele size range (bp)	T_a (°C)	Fluorescent label	GenBank accession no.
LH2	F: ACACCAACAATGACATCCTGC	$(AAAAG)_6$	187 M	59	NED	MK246216
	R: GTTTGAAGTACAGACCCAATACACC					
LH4	F: GGGAAATTGTGCGCTTAGTTTG	$(AAAAT)_6$	133 M	59	VIC	MK246217
	R: GTTTCACACTTAACACACCTTGCG					
LH10	F: GTTTCTTGCGAGGCCTTCTTTC	$(AAAG)_6$	126-146	59	FAM	MK246218
	R: TCGGGTTCAAATCATGGAATCC					
LH14	F: TTTCGGTAAGCAGGTTCCCATC	$(AAATAT)_6$	210-234	60	VIC	MK246219
	R: GTTTCTCTCCACTTTCCCAGAAAC					
LH16	F: GATGCCAACACAGGTAAACATC	$(AAATGT)_7$	225-243	59	NED	MK246220
	R: GTTTATACCGGCATAACTTTCGCC					
LH21	F: GTTTGTATCATCACACACAGTCGG	$(AACAAT)_9$	258-295	59	FAM	MK246221
	R: AGCCTGCCTATGATTGTACTCC					
LH22	F: ATGCCTCGTTGTTGATGGTC	$(AACAAT)_6$	203-305	59	VIC	MK246222
	R: GTTTCAAAGTGGGACTGGTAGC					
LH24	F: TGTGCTTGTTCCTGTTCCAG	$(AACAGG)_6$	137 M	59	FAM	MK246223
	R: GTTTAAACCGCATACTGTGAAAGATG					
LH25	F: GTTTAACCGTTTCTCCTAATCCGC	$(AACC)_6$	218-238	59	FAM	MK246224
	R: TGGAGACGAGTACCAGAACTAC					
LH67	F: TCCTATGTGATCCCTGTGTGTC	$(ATC)_{15}$	192-236	59	VIC	MK246225
	R: GTTTAAGGCTGTCTACGTCTTACCC					
LH68	F: AGGTTATCACGGTTTAGCGC	$(ATGC)_6$	121-133	60	PET	MK246226
	R: GTTTCCGGTCAGCATGTCTAC					
LH69	F: ATCTGGTGAAGGTGTGACTACC	$(CCG)_8$	181-208	59	PET	MK246227
	R: GTTTCAGAGGCAGAAGGTTTGG					
LH78	F: GTTTGTGCTTGCTCCCTAACAAC	$(AAC)_9$	185-244	59	NED	MK246228
	R: ATGACGTGATTGCTGCTGTG					
LH82	F: AAGCGCAAAGATTGTCCCAC	$(AAG)_{12}$	259-334	60	VIC	MK246229
	R: GTTTCATCAATCGGTTTCACGCC					
LH83	F: TGATCAAGGCCGGCATATTG	$(AAG)_{10}$	136-168	59	PET	MK246230
	R: GTTTAGAGAGTTGGATCAAGGACATG					
LH84	F: AAAGCATTGCGAGAAGAGGG	$(AAG)_{11}$	103-125	59	PET	MK246231
	R: GTTTAATAGCGCGCTGAAGAGTG					
LH89	F: GTTTCTTCTTCATCATGTCGCCTG	$(AATAT)_7$	137-211	59	PET	MK246232
	R: GGACAAATAACCGATCCGATCC					

Note: T_a= annealing temperature; M= Monomorphic

Table 2. Descriptive statistics for 14 polymorphic microsatellite markers in *L. helleri*

	Blue Ridge Parkway (n=36)				Linville (n=30)				Shortoff (n=20)			
Locus	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e
LH10	4	2.65	0.133*	0.622	3	1.16	0.143	0.135	4	3.60	0.133*	0.722
LH14	6	3.95	0.571	0.747	6	3.64	0.619*	0.726	5	2.75	0.579	0.636
LH16	5	3.07	0.567*	0.674	4	1.48	0.385	0.323	4	2.23	0.389	0.551
LH21	5	3.15	0.190*	0.683	3	2.13	0.000*	0.531	5	3.03	0.083*	0.670
LH22	2	1.09	0.000*	0.083	6	3.35	0.200*	0.701	3	2.77	0.308*	0.639
LH25	6	2.50	0.458*	0.601	5	3.81	0.792	0.738	5	3.93	0.500*	0.745
LH67	8	3.98	0.500	0.749	11	6.90	0.346*	0.855	4	3.06	0.200*	0.673
LH68	5	3.09	0.440*	0.676	5	4.02	0.133*	0.751	3	1.92	0.000*	0.480
LH69	7	3.17	0.379*	0.685	4	2.08	0.115*	0.518	4	2.34	0.158*	0.572
LH78	8	3.83	0.423*	0.739	5	4.25	0.318*	0.764	6	5.02	0.688*	0.801
LH82	17	10.00	0.667*	0.900	15	8.01	0.600*	0.875	9	4.78	0.789	0.791
LH83	3	2.07	0.833*	0.517	5	2.04	0.680	0.510	3	2.10	1.000*	0.525
LH84	6	2.87	0.464*	0.651	6	4.17	0.760	0.760	5	3.38	0.350*	0.704
LH89	5	2.26	0.280	0.557	5	2.10	0.250*	0.524	7	4.35	0.350*	0.770

Note: n = number of individuals sampled; A = number of alleles; A_e = effective number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; (*) indicate a significant deviation from HWE ($P<0.05$).

Table 3. Cross amplification of 17 primer pairs in other *Liatris* species.

Marker	*L. aspera*	*L. pycnostachya*	*L. microcephela*
LH2	100%	100%	100%
LH4	100%	100%	100%
LH10	-	-	-
LH14	100%	100%	100%
LH16	100%	100%	100%
LH21	60%	20%	-
LH22	100%	100%	100%
LH24	60%	60%	-
LH25	100%	100%	100%
LH67	60%	-	100%
LH68	-	-	-
LH69	100%	100%	100%
LH78	100%	100%	100%
LH82	100%	100%	100%
LH83	100%	100%	100%
LH84	100%	60%	100%
LH89	100%	100%	100%

Note: Percentage of 5 individuals that successfully amplified an appropriately sized product for the locus; (-) represents failure in amplification.

APPENDIX

Appendix 1. Voucher info for the specimens used in this study. All specimens are deposited in the I.W. Carpenter Jr. Herbarium at Appalachian State University (BOON).

*GPS coordinates are not provided in the interest of protecting locality information for this federally listed species.

Species	Population	# Samples Represented	Herbarium Accession no.	Collector
L. helleri	Shortoff	20	BOON28016	P. Sullins & G. Kauffman
L. helleri	Linville Gorge	30	BOON28017	P. Sullins
L. helleri	Blue Ridge Parkway	36	BOON28026	P. Sullins
L. aspera	Gardens of the Blue Ridge	5	BOON30483	L. Clark
L. microcephela	Gardens of the Blue Ridge	5	BOON30484	L. Clark
L. pycnostachya	Gardens of the Blue Ridge	5	BOON30485	L. Clark

Chapter 3

Delineation of Species Boundaries Using Microsatellite Loci in the Federally Listed Perennial Herb *Liatris helleri* Porter (Asteraceae)

Logan C. Clark and Matt C. Estep

Department of Biology, Appalachian State University, 572 Rivers Street, Boone NC, 28607-2027 USA

Email addresses: LCC: clarklc@appstate.edu

MCE: estepmc@appstate.edu

Number of words:

Manuscript received_____; revision accepted_____

Author for correspondence: estepmc@appstate.edu

ORCID ID: 0000-0002-4852-591X

Acknowledgements

I would like to thank to Kris Fraizer, Marietta Shattelroe, and Luke Appling for their assistance in field collections. Thank you to Dr. Howard Neufeld and Dr. Jen Rhode Ward for their help in the editing process. The United States Fish and Wildlife Service (F16AP00773) and the Appalachian State University Office of Student Research helped fund this research. The North Carolina Natural Heritage Program provided locality information, for which I am grateful. Ron Lance and Nora Murdock provided insight on past augmentation events. Thank you to Chris Ulrey (NPS) and Gary Kauffman (USFS) for their help and guidance. Thank you to the US Fish and Wildlife Service (TE178876-1), NC State Parks (2017_0165), NC Plant Conservation Program (#497), NC Wildlife Resources Commission, The Nature Conservancy, National Park Service (SHEN-2018-SCI-0002), and West Virginia Department of Natural Resources (P01-2018) for providing permits to sample these populations.

Abstract

Liatris helleri Porter is a perennial herb endemic to high elevation rock outcrop communities in northwest North Carolina that has been protected under the Endangered Species Act since 1987. The degree of relation between *Liatris helleri* Porter and its conger *Liatris turgida* Gaiser has become a point of taxonomic contention. Previous morphological and genetic studies have suggested the possibility that these taxa do not fall discretely into separate taxonomic treatments, which has led to unclear species boundaries. The current solution for this taxonomic issue has been to subsume *L. turgida* populations under the *L. helleri* epithet, with a possibility that these taxa are separable at a varietal level. This has brought *L. helleri*'s status of federal protection into question and resolution of this issue is needed to provide land managers and conservationists with accurate delineations of these taxa. This study provides high-resolution microsatellite data to address the degree of relation between *L. helleri* and *L. turgida*. The results of this study identify a genetically distinct metapopulation of *L. helleri*, areas of admixture, and genetic diversity estimates for both species. Based on these findings we argue for the continued protected status of *L. helleri* (with a reduced range) and for a reevaluation of the protected status of *L. turgida* in North Carolina.

Keywords: conservation, *Liatris helleri; Liatris turgida*; population genetics**;** rock outcrop; species delineation**;** southern Appalachians

Introduction

Liatris helleri Porter is a perennial herb endemic to high elevation rock outcrop communities, 1200 m or more above sea level, in northwest North Carolina that has been protected under the Endangered Species Act since 1987. This species faces threats from outdoor recreation, natural succession, and possibly climate change (Murdock, 2000). Since federal listing of this species USFWS recovery plans have been undertaken in efforts to preserve the species. These involve collaborations with the US Forest Service, the National Park Service, and the NC Plant Conservation Program in the form of continual long-term monitoring projects, successful augmentations of over 3,000 individuals to natural populations, educational outreach to land managers and recreational

organizations, and prescribed burns at several localities to eliminate competition from other plant species and to increase recruitment (Murdock, 2000; C. Ulrey personal comm.).

Species delimitation within the genus *Liatris* has been considered one of "unusual difficulty" (Gaiser 1946). The current taxonomy describes 40-50 species of *Liatris*, primarily confined to the eastern seaboard of North America (Gaiser 1946; Nesom 2005; Weakley 2015). Species of *Liatris* are broadly sympatric, but ecologically distinct in their distributions, which are related to gradients of available nutrients, soil moisture, and elevation. The ease of hybridization in this genus has likely been facilitated by overlap in seasonal phenology between species and similar chromosome morphology between taxa, which may contribute to the lack of well-defined species boundaries (Gaiser 1950; Levin 1967). Gaiser (1950) investigated the cytotaxonomy of the genus *Liatris* and found *L. helleri* and *L. turgida* were most similar to each other, but distinguished from each other based on ecological preference and the shallow corm of *L. helleri*.

Liatris helleri was previously distinguished from its congener *L. turgida* Gaiser based on pappus length (Nesom 2005). Godt and Hamrick (1996) employed allozyme markers to evaluate genetic diversity and population structure within this species, but did not address its relation to *L. turgida*. These authors found unusually high genetic diversity within populations, stating *L. helleri* to be more comparable to its widespread conger *L. cylindracea* Michx. Two explanations were offered to account for the high genetic diversity: 1) this species was hybridizing with other species despite displaying evidence of genetic isolation or 2) *L. helleri* once had a more widespread range with larger populations (Godt and Hamrick 1996). Nesom (2005) proposed a recircumscription of this taxa based on findings of inconsistent morphology to separate *L. helleri* from *L. turgida.* Nesom also cited the findings of higher genetic diversity than would be expected for an isolated endemic, such as *L. helleri* (Godt and Hamrick 1996). The proposed changes in taxonomy of *L. helleri* and *L. turgida* would effectively expand the range of *L. helleri* from northwest North Carolina throughout central Appalachia (Figure 1). If the suggested changes in taxonomy are accepted, then the protected status of *L. helleri* under the Endangered Species Act may be brought into question.

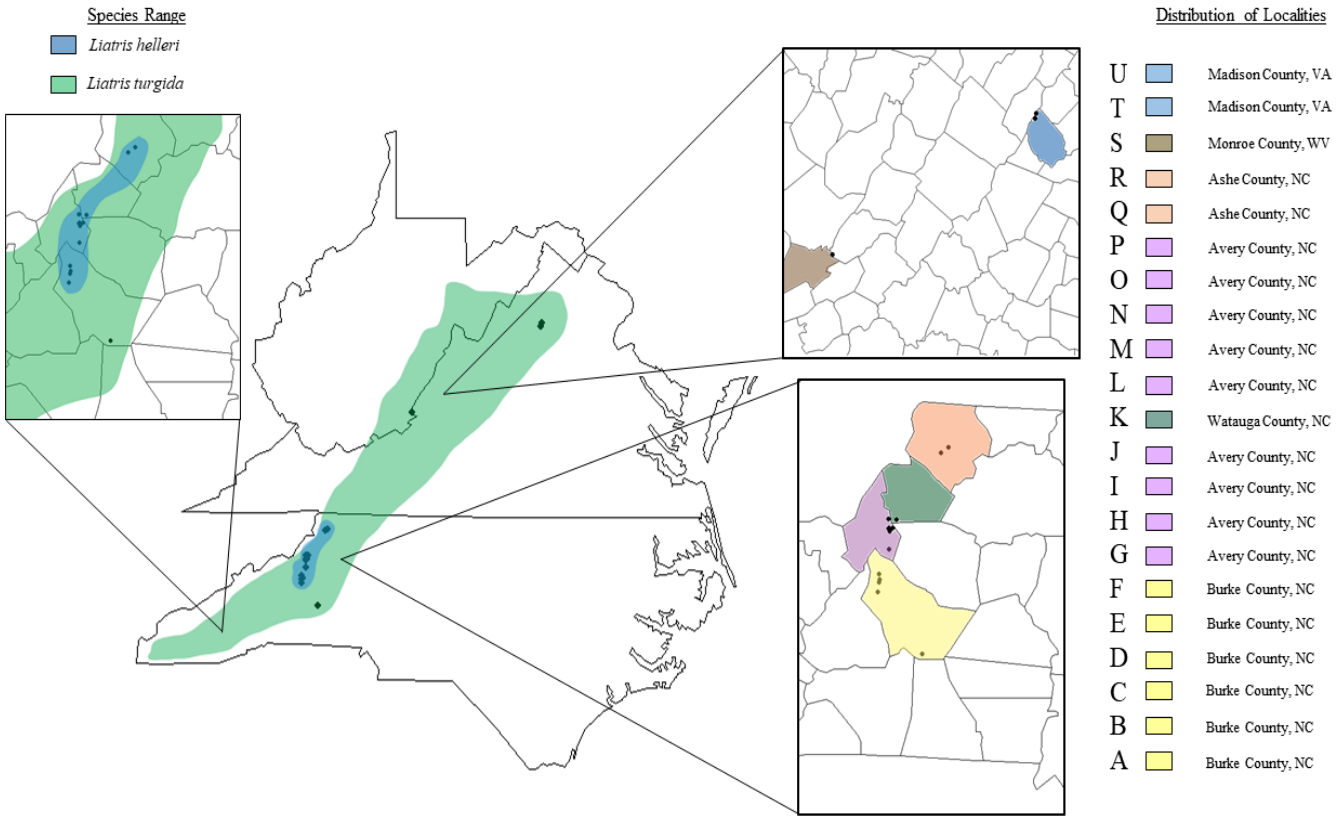


Fig. 1 Map of sampling sites and species ranges. Black dots represent populations from which plants were taken. The blue shaded region represents *L. helleri*, and the green shaded region represents *L. turgida*. Populations are coded A-U, organized approximately south to north, and labeled by color to represent counties. Specific locality information has been masked in the interest of confidentiality for this federally listed species.

This study employs a series of 12 microsatellite markers developed for the genus *Liatris* to investigate the species boundaries between these two taxa (Clark et al. 2019). Microsatellite markers provide the most cost effective and efficient method currently available to address issues of speciation at the population level, especially for rare taxa (Vieira et al. 2016). The separation of *L. helleri* and *L. turgida* has proven to be problematic, but by implementing a microsatellite analysis, this study provides a population level delineation of these two taxa. The results of my study suggest that a pocket of populations within the traditional range of *L. helleri* have maintained a genetically distinct metapopulation. I also have found areas of admixture and populations interpreted to represent *L. turgida*. The data generated from this research can provide land managers and conservationists with

the necessary information for them to make informed management decisions for this Federally Endangered species.

Materials and Methods

Field Sampling

Samples were collected from mid-July through August in 2016 and 2018. Plants were considered separate individuals if they occurred in separate clumps separated by a distance of at least 10 cm. Single leaf tissue samples from 20 individuals were taken and stored in cryovials with silica gel and then placed into a -20°C freezer until needed for downstream analyses. In some cases, less than 20 individuals occurred in the population, resulting in fewer sampled individuals. Twenty samples across the locality should adequately capture the allelic diversity for that locality and in populations consisting of less than 20 individuals reflect total allelic diversity at the selected microsatellite loci (Hale et al. 2012). A total of 21 localities were sampled, representing the range of both *L. helleri* and *L. turgida* (Fig. 1). Maps were generated in R Studio (RStudio Team, Boston, MA, 2015) using the package *maps* (version 3.3.0; Becker et al. 2016).

DNA Extraction and Genotyping

DNA was extracted from 288 individuals and 12 microsatellite loci were amplified using techniques and primers described in Clark et al. (2019). These markers included (LH2 – LH84). Individuals were arrayed into four 96-well plates at 10-30 ng/µl with duplicated individuals to act as controls across plates. PCR products were fluorescently tagged with VIC, FAM, PET, or NED and multiplexed after amplification (Invitrogen Carlsbad, CA, USA). Fragment analysis was conducted at the Georgia Genomics and Bioinformatics Core (Athens, Georgia, USA) on an ABI 3730xl sequencer. Resulting chromatograms were scored and assessed in Geneious (Kearse et al. 2012). Thirty-nine individuals representing 2 populations from Clark et al. (2019) were included in the dataset. The data were then checked for scoring error and null alleles with Microchecker (Van

Oosterhout et al. 2004). Marker LH21 displayed evidence of nulls alleles (Oosterhout = 0.3681). Each marker was also examined via a PCA in Genalex to ensure consistent clustering of populations and to identify outliers. A group of 24 individuals failed to consistently amplify across all loci and were removed from the dataset for downstream analyses. The final data set included 303 individuals from 21 localities.

Microsatellite Analysis

Basic diversity statistics: average alleles per locus, average effective alleles per locus, private alleles per population, per locus Hardy Weinberg Equilbrium (HWE), Mantel's Test (999 permutations), and pairwise F_{ST} and R_{ST} (999 permutations) were calculated in Genalex (Peakall and Smouse, 2006). Pairwise G^1_{ST} and Jost's D values between populations were calculated using *mmod* (Winter 2012). Allelic richness averages per locus we calculated using the *PopGenReport* package in R (Adamack et al., 2014). A Discriminant Analysis of Principal Components (DAPC) was generated using *adegenet* (Jombart 2008; Jombart et al. 2010). Clustering analyses were performed in STRUCTURE (Pritchard et al. 2000) Initial parameters were set to 50,000 burn-ins with an MCMC chain of 500,0000, using an admixture model, correlated allele frequency model, and run on K values 1-25 with 5 iterations for each K value. Resulting files were compressed and input into STRUCTURE HARVESTER (Earl and vonHoldt 2012) to determine delta K using the Evanno Method. K=2 and K=4 were further examined and separate STRUCTURE analyses were run again for each K value with 100,000 burn-ins and an MCMC chain of 1,000,000. Resulting ancestry coefficient values were plotted in bar plots using STRUCTURE PLOT (Ramasamey et al. 2014). R Studio (RStudio Team, Boston, MA, 2016) was used to perform all other analyses. Raster maps displaying ancestry coefficients were generated in *LEA* (Frichot and François 2015; François 2016). Genalex files were read into the R environment using *poppr* (Kamvar et al. 2014). Cavalli-Sforza Chord distances were generated in *hierfstat* (Goudet 2005). A neighbor-joining tree was generated from the chord distances using *ape* (Paradis et al. 2004).

Results

Descriptive statistics

A total of 135 alleles were identified across all loci with amplified product sizes ranging from 100 bp to 329 bp (Table 1). The number of alleles per locus for polymorphic loci ranged from 11 to 35 with an average of 15. Average effective alleles per locus ranged from 1.827 (LH68) to 4.860 (LH82) with an average of 2.668. Allelic richness per locus ranged from 1.346 (LH68) to 1.810 (LH82) with an average of 1.555. Observed heterozygosity per locus ranged from 0.087 (LH21) to 0.706 (LH82) with an average of 0.319. Expected heterozygosity per locus ranged from 0.330 (LH68) to 0.77 (LH82) with an average of 0.395. There are 3 significant deviations from HWE: LH21 ($P<0.05$), LH67 ($P<0.05$), LH68 ($P<0.01$). F_{ST} values per locus ranged from 0.177 (LH82) to 0.491 (LH21) with an average of 0.318.

Table 1 Summary of diversity statistics for 9 loci. Monomorphic markers LH2, LH4, and LH24 were excluded from this table.

Locus	Size Range	N_a	N_e	A_R	H_o	H_e	HWE	F_{ST}
LH14	208-238	11	2.456	1.557	0.466	0.531	ns	0.319
LH16	219-258	10	2.095	1.502	0.507	0.483	ns	0.282
LH21	214-295	12	1.960	1.446	0.087	0.405	*	0.491
LH25	200-242	15	2.907	1.628	0.582	0.592	ns	0.270
LH67	192-248	19	2.749	1.581	0.433	0.549	*	0.261
LH68	114-136	11	1.827	1.346	0.102	0.330	**	0.574
LH69	169-226	11	2.800	1.585	0.510	0.562	ns	0.244
LH82	257-329	35	4.860	1.810	0.706	0.771	ns	0.177
LH84	100-157	11	2.354	1.539	0.439	0.513	ns	0.244
Mean		15	2.668	1.555	0.319	0.395	-	0.318

Size Range = amplified product size range (bp). N_a= Number of alleles observed per locus.
N_e= Number of effective alleles per locus. A_R= Averaged allelic richness per locus.
H_o and H_e = heterozygosity observed and heterozygosity expected. Significant deviations from HWE (Key: ns=not significant, * $p<0.05$, ** $p<0.01$) and average F_{ST} values.

Genetic Clustering

The DAPC results displayed a central cluster consisting of the majority of populations (Fig. 2). Populations A and U, respectively representing the southern and northern most populations, both grouped separately from the main cluster. Populations Q and R, representing the amphibolite mountain macrosite populations, also clustered separately from the central cluster. Population J, representing the Blue Ridge Parkway, also displayed a distinct cluster.

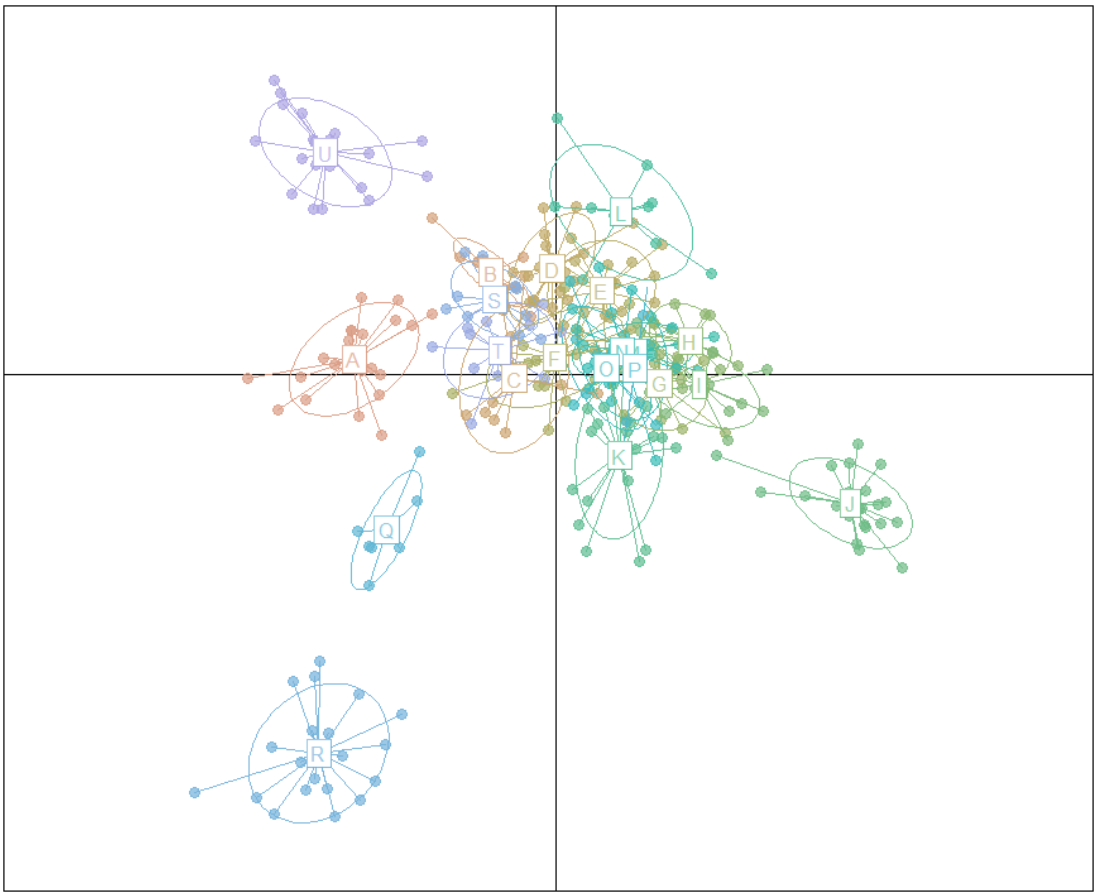


Fig. 2 Discriminant Analysis of Principle Components (DAPC) made in *adegenet*. Each circle labeled and letter corresponds to a population, and each dot represents an individual plant.

Results from STRUCTURE HARVESTER indicated the highest likelihood for K=2 (ΔK= 97.15). There were also peaks at K=4 (ΔK= 6.65), K=6 (ΔK= 22.69), K=11 (ΔK= 21.51), and K=13 (ΔK= 12.36) (A1). All K values that displayed a peak were investigated and mapped, but K=2 and

K=4 were chosen as the most informative due to the high statistical support (K=2) and biological relevance for management implications (K=4). A bar plot displaying ancestry coefficients of K=2 identified distinct groupings (Fig. 3a). Populations A, B, C, and D all displayed the highest ancestry coefficient values with Ancestral Group 1 (AG1) while populations E and F displayed more admixture with Ancestral Group 2 (AG2). Populations G, H, I, and K displayed the highest degree of relation to AG2. Populations L, M, N, O, and P were identified as sharing a high percentage of relation to AG2, but also admixed with AG1. Populations Q, R, S, T, and U displayed predominately AG1 ancestry. The raster map displaying the ancestry coefficients for K=2 made in *LEA* across the landscape also reflect the STRUCTURE results (Fig. 3c). A bar plot of ancestry coefficients for K=4 provided higher resolution of the sub-structuring of AG1 from the K=2 analysis (Fig. 3b). This analysis identified populations A, B, and C as sharing the highest relation with populations S, T, and U. Populations E, D, and F represented a distinct cluster within the data, sharing some relation with populations B and C. This analysis identified the same group of populations H, I, J, and K as retaining the highest percentage of AG2 ancestry, while population G displayed higher admixture than in K=2. This admixture was most closely shared with populations D, E, and F, which represent populations in Burke County. A raster map displaying the ancestry coefficients of K=4 across the landscape can be found in Fig 3d.

Based on the traditional range and habitat of *L.helleri* and the distinct grouping of populations G, H, I, J, and K, which all displayed the highest relation to AG2, AG2 ancestry will be referred to throughout the remainder of the paper as *L. helleri* ancestry and AG1 will represent *L .turgida* ancestry.

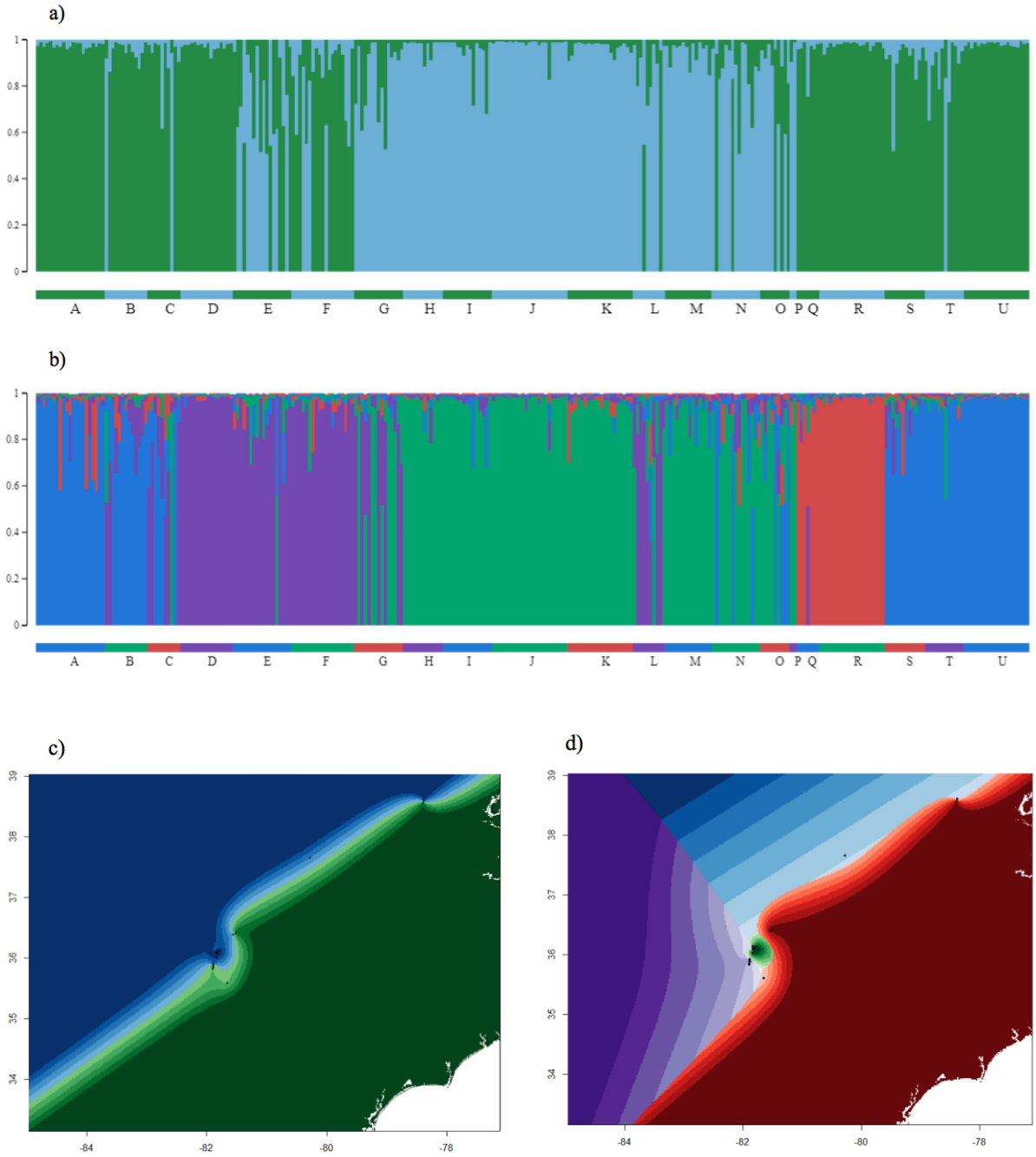


Fig. 3 (a&b) Bar plot of STRUCTURE results of K=2 and K=4 respectively. Colored blocks along x-axis correspond to population size and each vertical bar represents an individual. (c&d) Map of North Carolina, Virginia, and West Virginia, displaying estimated Ancestry Coefficients of K=2 and K=4 respectively. In K=2, the blue represents *L. helleri* ancestry. In K=4, the green represents *L. helleri* ancestry.

Neighbor joining trees were generated using F_{ST} values and Cavalli-Sforza chord distance (D_{ch}). Both methods resulted in the same groupings, but with slightly different arrangements and branch lengths. D_{ch} were chosen as the most appropriate distance to build the NJ tree (Takezaki and Nei 1996). The resulting tree displayed four distinct clades: one representing traditional *L. helleri* populations (pink), two representing populations experiencing admixture to varying degrees (blue), and one representing *L. turgida* (green) (Fig. 4). Populations in the *L. helleri* clade (G, H, I, and J) correspond to populations along the Blue Ridge Parkway. Grouping C, D, E, F, and L primarily represents those populations in Burke County NC along with a single population from Avery County NC (L), suggesting these populations were in recent contact. Grouping K, M, N, O, and P represents those populations in Avery County consisting of localities along the northern side of Grandfather Mountain or its summits. Grouping A, B, Q, R, S, and U represent those populations traditionally considered to be *L. turgida*. There is some evidence of distinct genetic clusters in populations representing the amphibolite mountain macrosite (Q and R) and populations representing Shenandoah National Park (T and U). Populations A, B, and S represent a sub-clade composed of the southernmost populations in Burke County, NC (A and B) with that from Monroe County, WV (S) a geographic distance of over 200 miles.

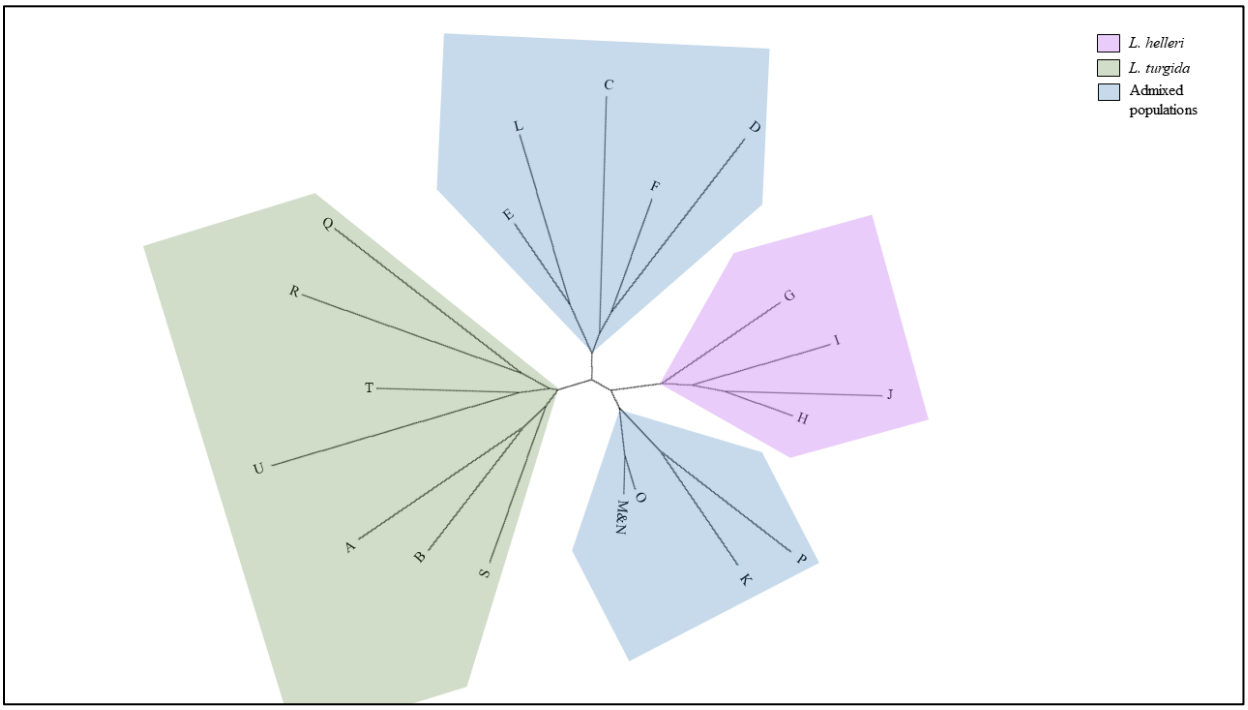


Fig. 4 Neighbor-joining tree made using Cavalli-Sforza chord distance (D_{ch}) made in *heirfstat* and *ape*. The purple clade represents true *L. helleri* populations. Blue clades represent admixed populations; note that*M&N are the same locality, representing separate areas within that site. The green clade represents *L. turgida* populations.

Genetic Distance

F_{ST} values between population pairs averaged 0.181 and ranged from 0.023 (N and O) to 0.390 (K and Q) (Table 2). Cavalli-Sforza chord distance (D_{ch}) values averaged 0.461 between population pairs and ranged from 0.1 (N and O) to 0.706 (Q and C & Q and D). Results between the two metrics were relatively consistent with the highest values being observed between populations geographically separated and those considered to represent separate taxa. The lowest values were observed between populations close to each other geographically or those representing the same species. There was no significant correlation of genetic distance vs. geographic distance indicated through a Mantel's Test ($R^2 = 0.0184$, $p = 0.001$;data not presented). The D_{ch} values can also been viewed in a graphic representation on a Neighbor-joining tree (Fig. 4).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	
A	0	0.370	0.602	0.560	0.475	0.502	0.563	0.563	0.552	0.592	0.568	0.586	0.516	0.455	0.445	0.550	0.532	0.522	0.406	0.461	0.546	**A**
B	0.092	0	0.437	0.406	0.423	0.440	0.459	0.484	0.485	0.601	0.496	0.561	0.460	0.368	0.333	0.538	0.540	0.530	0.369	0.410	0.526	**B**
C	0.165	0.150	0	0.496	0.401	0.399	0.575	0.671	0.606	0.704	0.625	0.592	0.482	0.416	0.457	0.648	0.706	0.515	0.485	0.528	0.598	**C**
D	0.145	0.110	0.190	0	0.350	0.350	0.485	0.612	0.573	0.647	0.591	0.510	0.558	0.496	0.505	0.639	0.706	0.557	0.548	0.627	0.625	**D**
E	0.110	0.100	0.145	0.104	0	0.287	0.342	0.371	0.502	0.383	0.439	0.285	0.343	0.358	0.345	0.449	0.593	0.541	0.421	0.428	0.537	**E**
F	0.153	0.144	0.145	0.139	0.094	0	0.320	0.422	0.522	0.501	0.499	0.421	0.368	0.335	0.365	0.490	0.519	0.418	0.476	0.500	0.526	**F**
G	0.168	0.135	0.245	0.192	0.102	0.116	0	0.222	0.359	0.391	0.386	0.424	0.384	0.408	0.390	0.475	0.536	0.546	0.550	0.507	0.533	**G**
H	0.175	0.168	0.267	0.243	0.131	0.164	0.064	0	0.242	0.240	0.355	0.348	0.286	0.307	0.285	0.403	0.454	0.565	0.504	0.408	0.598	**H**
I	0.196	0.195	0.288	0.268	0.204	0.224	0.165	0.102	0	0.365	0.444	0.544	0.295	0.297	0.322	0.462	0.569	0.561	0.477	0.401	0.526	**I**
J	0.203	0.219	0.281	0.285	0.177	0.226	0.180	0.115	0.165	0	0.432	0.384	0.437	0.435	0.436	0.486	0.588	0.631	0.550	0.548	0.700	**J**
K	0.247	0.246	0.340	0.314	0.204	0.271	0.242	0.204	0.248	0.237	0	0.404	0.399	0.376	0.333	0.315	0.570	0.429	0.518	0.477	0.602	**K**
L	0.210	0.220	0.251	0.224	0.093	0.193	0.168	0.163	0.300	0.239	0.254	0	0.467	0.404	0.413	0.452	0.516	0.614	0.494	0.548	0.584	**L**
M	0.173	0.152	0.183	0.223	0.142	0.126	0.179	0.182	0.165	0.235	0.263	0.288	0	0.133	0.131	0.265	0.589	0.516	0.406	0.328	0.543	**M**
N	0.126	0.106	0.151	0.192	0.125	0.128	0.167	0.145	0.140	0.190	0.201	0.221	0.046	0	0.100	0.246	0.538	0.432	0.322	0.324	0.524	**N**
O	0.120	0.089	0.161	0.185	0.124	0.156	0.180	0.155	0.146	0.203	0.221	0.233	0.066	0.023	0	0.288	0.468	0.436	0.280	0.244	0.549	**O**
P	0.235	0.223	0.300	0.313	0.222	0.255	0.258	0.231	0.269	0.268	0.216	0.321	0.158	0.110	0.138	0	0.681	0.591	0.478	0.473	0.719	**P**
Q	0.219	0.192	0.351	0.303	0.230	0.284	0.225	0.192	0.265	0.299	0.390	0.297	0.332	0.254	0.215	0.408	0	0.488	0.450	0.471	0.571	**Q**
R	0.161	0.165	0.210	0.206	0.168	0.167	0.211	0.208	0.227	0.251	0.273	0.289	0.201	0.168	0.173	0.330	0.255	0	0.504	0.459	0.571	**R**
S	0.122	0.101	0.171	0.193	0.140	0.192	0.219	0.204	0.179	0.254	0.289	0.245	0.142	0.093	0.067	0.213	0.214	0.190	0	0.354	0.520	**S**
T	0.150	0.143	0.184	0.222	0.160	0.187	0.218	0.191	0.173	0.261	0.263	0.268	0.136	0.097	0.085	0.219	0.232	0.190	0.116	0	0.419	**T**
U	0.190	0.191	0.237	0.231	0.190	0.180	0.214	0.232	0.238	0.286	0.332	0.296	0.204	0.197	0.204	0.362	0.296	0.191	0.208	0.163	0	**U**
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	

Table 2 Pairwise F_{ST} values between populations below diagonal. Pairwise Cavalli-Sforza chord distance (D_{ch}) values between populations above diagonal.

Genetic Diversity

Populations representing *L. helleri* displayed an average of 39.5 alleles per population ranging from 35 (J) to 43 (G), an average number of effective alleles per locus of 2.243 ranging from 2.071 (I) to 2.595 (G), an average of 1.250 private alleles with a maximum of 2 (I), and observed heterozygosity ranging from 0.280 (I) to 0.342 (G) (Table 3). Admixed populations displayed an average of 34.5 alleles per population ranging from 5 (O) to 44 (F&N), an average of 2.192 effective alleles per locus with a range of 1.422 (P) to 2.746 (O), an average of 1.400 private alleles per population with up to 6 (E), and an average of 0.311 observed heterozygosity with a range from 0.208 (P) to 0.400 (E). The observed range of allelic diversity values are likely due to unequal sampling sizes; e.g., population O had only 2 individuals. *L. turgida* populations displayed an average of 28.571 alleles per population ranging from 12 (A&B) to 41 (S), an average of 2.339 effective alleles per population ranging from 1.673 (Q) to 3.111(B), an average of 3.143 private alleles per population with up to 7 (A), and an average observed heterozygosity of 0.337 ranging from 0.305 (Q) to 0.363 (S).

Table 3 Summary of diversity statistics for populations separated by clades based on Neighbor-joining tree.

L. helleri

Population	N	N_a	N_e	P_a	H_o	H_e
G	15	43	2.595	1	0.342	0.435
H	12	42	2.163	1	0.330	0.414
I	15	38	2.071	2	0.280	0.358
J	23	35	2.141	1	0.285	0.385
Mean		39.500	2.243	1.250	0.309	0.398

Admixed Populations

Population	N	N_a	N_e	P_a	H_o	H_e
C	10	35	2.249	1	0.314	0.402
D	16	43	2.291	4	0.327	0.419
E	18	55	2.837	6	0.400	0.489
F	19	44	2.338	1	0.321	0.414
K	20	31	1.614	0	0.216	0.245
L	10	28	1.839	1	0.235	0.318
M	14	41	2.170	1	0.388	0.376
N	15	44	2.411	0	0.329	0.429
O	9	5	2.746	0	0.374	0.451
P	2	19	1.422	0	0.208	0.219
Mean		34.500	2.192	1.400	0.311	0.376

L. turgida

Population	N	N_a	N_e	P_a	H_o	H_e
A	21	12	3.076	7	0.343	0.524
B	13	12	3.111	3	0.313	0.519
Q	7	30	1.673	3	0.305	0.302
R	20	36	2.034	1	0.347	0.416
S	12	41	2.295	3	0.363	0.408
T	12	36	2.153	2	0.348	0.390
U	20	33	2.035	3	0.338	0.372
Mean		28.571	2.339	3.143	0.337	0.419

N = number of individuals. N_a = number of alleles per population. N_e = average number of effective alleles over loci per population. P_a = private alleles per population. H_o and H_e = heterozygosity observed and heterozygosity expected.

Discussion

Sampling and Loci

Adequate sampling throughout the breadth of the circumscribed species range was achieved (Nesom 2005) and samples failing to amplify across 6 or more markers were removed from the dataset. Marker LH21, which indicated possible evidence of null alleles, was not removed from the data due to previous findings of low heterozygosity in *L. helleri* (Godt and Hamrick 1996). Three of the markers used (LH2, LH4, and LH24) were monomorphic, but were run to investigate if *L. turgida* populations would display a different allele than *L. helleri*. Although they did not, they remained in the dataset. LH82 displayed the highest diversity (35 alleles), while the other markers ranged from 10-19 alleles. Therefore, LH82 may provide the most informative data out of the developed markers, but the other markers employed were still able to capture an adequate representation of the allelic diversity. The resulting data set encompasses 303 individuals genotyped across 12 markers, of which 9 were polymorphic and 3 monomorphic.

Genetic Diversity and Distance

The observed genetic diversity in this analysis is consistent with previous findings of high allelic diversity and low heterozygosity (Godt and Hamrick 1996). Lower observed heterozygosity than expected was consistently observed across populations except for M and Q. This could indicate low outcrossing rates across populations of both taxa. Populations considered *L. helleri* in this study display higher allelic diversity than those populations considered *L. turgida* despite *L. turgida* populations being more numerous. This lends support to the Godt and Hamrick (1996) hypothesis that *L. helleri* was once a more widespread species that has become restricted in recent times. The higher diversity supports *L. helleri*'s potential status as a post-Pleistocene alpine relic species, like many other high elevation rock outcrop community members in the southern Appalachians (Ulrey et al. 2016; Wiser et al., 1996). *L. helleri* populations also retained approximately half of the average number of private alleles observed in *L. turgida* populations, meaning that these populations are

likely experiencing genetic drift which is leading to the fixation of certain alleles. These findings would also align with those observed by Godt and Hamrick (1996). The isolation of these populations is also reinforced by F_{ST} values. Thresholds of approximately 0.15 to 0.25 are thought to indicate high differentiation and could be used to argue for distinct species ranking (Balloux and Lugon-Moulin 2002). Values within or exceeding this threshold were observed between population pairs consisting of those populations interpreted to represent *L. helleri* and *L. turgida*. This lends further evidence to the separation of these taxa.

Genetic Clustering

The highest likelihood for K clusters was 2 ($\Delta K = 97.15$), and these two clusters have been interpreted to represent *L. helleri* Porter (AG2) and *L. turgida* Gaiser (AG1). Populations G, H, I, J, K, M, and P seem to represent the purest populations of *L. helleri* based on the STRUCTURE analysis for K=2. These populations are not without some introgression of *L. turgida*, but instead represent the best remaining examples we have of *L. helleri*. These populations consist of augmented and unaugmented populations (NCNHP, 2019), meaning that some previous augmentation efforts have been successful in maintaining the genetic integrity of the target population. Populations A, B, C, D, Q, R, S, T, and U all show moderate to strong affinity toward the *L. turgida* gene pool. Populations E and F at the more southern and lower elevation portion of the range display evidence of admixture between the taxa. Populations L, N, and O also display evidence of admixture, but to a lesser degree. This is likely due to previous augmentation efforts diluting the gene pool, but close geographic location of these populations to those representing *L. helleri* may have permitted some gene flow. These admixed populations within the private park at the top of Grandfather Mountain may also pose threats of genetic introgression to the pocket of populations identified as *L. helleri* occurring on the slopes below or those further along the ridge that have remained more isolated from augmented localities.

K=4 (ΔK= 6.65) was also chosen for further investigation to examine a finer scale resolution to gene flow. In this analysis the amphibolite mountain macrosite populations (Q and R) pulled out as a distinct group, most closely sharing relations with population A at the southern-most end of the species range in Burke County, NC. Populations representing the Linville Gorge mountaintop populations D, E, and F also pulled out as a distinct grouping, most closely sharing relations with lower elevation localities in Burke County, NC and population G in Avery County. These results are supported in the NJ tree (Fig. 4). This analysis also identified H, I, J, K, and P as predominately *L. helleri* populations. Population G, unlike in the K=2 analysis, displayed moderate levels of admixture with the Linville Gorge populations. This is logical since population G is closer in geographic proximity to populations D, E, and F than grouping H, I, J, and K. Populations Q and R represented a distinct cluster in the data and appear to share a small degree of gene flow with populations A, B, and C. These results are corroborated in the raster map displaying the ancestry coefficients of K=4 across the landscape (Fig. 3d). Population L, appears to be most closely related to those populations from the Linville Gorge despite is closer geographic proximity to populations H-P. The divide between those populations on the approximately north and south facing slopes seems to create a barrier to gene flow between these taxa.

Management and Conservation Implications

Based on evidence from the data generated in this study, populations G, H, I, J, and K represent populations of true *L. helleri*. This would warrant a range reduction in those populations considered *L. helleri* to a small area along the southern slopes of Grandfather Mountain and nearby areas. This would also warrant the taxon's continued protection status under the Endangered Species Act. Those populations experiencing admixture, especially M, N, O, and P, should continually be monitored; this is because they still retain some *L. helleri* genetic identity and are likely contributing some gene flow to true *L. helleri* populations due to their close geographic proximity. Concerns of introgression from these populations may also need to be addressed by local land managers.

This study also argues for a reevaluation of the protective status of *L. turgida* in all states in which it occurs, but specifically in North Carolina. During the fieldwork performed for this study neither species was widespread or commonly occurring in natural populations. Three distinct segments of population structure were identified in *L. turgida* using the K=4 analysis. The analysis of K=6 displayed similar results, with further substructuring of those populations interpreted to represent *L. turgida*. The substructuring observed in K=6, K=11, and K=13 did not provide biological relevance toward management actions or high statistical support. Population grouping D, E, and F, grouping A, B, C, S, T, and U, as well as grouping Q and R may represent evolutionary significant units of conservation in *L. turgida*.

Locally sourced augmentations have taken place at several localities in the past (NCNHP, 2019; G. Kauffman per. comm.) These augmentations appear to be successful at several sites including populations H and J. This species is known to grow well in greenhouse conditions, but during previous augmentations there were issues with corms being too large for planting in shallow soil due to growth in 1-quart pots (R. Lance per. comm.). Future augmentation should seek to grow plants in shallow soiled containers to facilitate shallow corm growth and use only locally sourced seeds for each population. Introduction of *L. turgida* into the gene pool of these populations could cause the loss of the *L. helleri* genetic identity. Introduction of plants from different localities within the H, I, J, or K grouping of populations could act to increase outcrossing in this species, but should be done with the upmost care to reduce contamination of the gene pool or loss of local adaptation.

Based on observations made during fieldwork, some of these localities may benefit from the establishment of a prescribed fire regime. Species of *Liatris* are known to respond well to fire management strategies (Medve 1987). This approach has been used successfully to increase recruitment for at least one locality (C. Ulrey, per. comm.). During field observations there were several localities within the G, H, I, J, and K grouping that were experiencing natural succession leading to subsequent shading and overgrowth of *L. helleri*. If we are to manage these habitats for

fire-dependent species such as *Liatris* then prescribed burns need to become more prevalent in our management practices.

This species also shares its home with some of the most sought-after and picturesque views in the southern Appalachians. These localities are among some of the most trafficked stretches of the Blue Ridge Parkway and are visited by rock climbers, tourists, and hikers who all pose threats of trampling to this species. Studies of recreations impact in this species have shown significant declines even after the installation of a boardwalk to minimize trampling (Sutter et al. 1993). Management for these populations should include educational public outreach, increased signage, and limiting access to these populations as there are only a small number left so every individual matters.

Conclusion

The results of this study have successfully identified true populations of *L. helleri.* This reaffirms the separation of *L. helleri* and *L. turgida* as distinct taxa based on genetic data. These findings suggest a further reduction in the number of populations considered to be *L. helleri* than previously suggested. Morphology does not appear to distinguish between these taxa, as is true for many other species of *Liatris* (Nesom 2005). The microsatellite markers implemented in this study have provided high resolution in the population structure and genetic relation of these taxa. Future studies should work to identify ecological and other abiotic factors contributing to the fine scale endemism exhibited in this species. Populations considered *L. helleri* by this study occur predominately within an area along the southern slopes of Grandfather Mountain known as the Boone Fork Bowl. Genetic analyses of another species occurring in this same area, *Geum geniculatum*, show a similar population structure on Grandfather Mountain (M. Shattelroe, unpublished data). There may be some abiotic or ecological variable that contributes to this structuring, which should be investigated in future studies.

The classification of species does not always fit neatly into predefined categories and not all genera exhibit explicit distinguishing morphological characters, but our classification of these

biological entities using molecular genetic techniques is extremely meaningful because of how we can more easily delineate species in the wild and to develop management plans for conserving them and protecting our natural resources. This study does not fully resolve the taxonomy of these taxa, but instead offers genetic data that identifies a distinct metapopulation of what has been interpreted to represent *L. helleri*. Taxonomists will have to decide specific ranking for *L. helleri*, whether that be full species status or subspecies ranking, while taking these finding into account. It is the aim of this study that land managers and conservationists apply the findings generated from this data in their pursuit to preserve this southern Appalachian and North Carolina endemic species.

References:

Adamack AT, Gruber B (2014) PopGenReport : simplifying basic population genetic analyses in R. Methods in Ecology and Evolution 5: 384–387

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. *Mol. Ecol.* 11: 155–165

Becker R, Wilks A, Brownrigg R, Minka T, Deckmyn A (2016) maps: Draw Geographical Maps. R package version 3.3.0

Cavalli-Sforza LL, Edwards AW (1967) Phylogenetic analysis. Models and estimation procedures. Am J Hum Genet 19:233–257

Clark LC, Estep MC (2019) Development of Seventeen Microsatellite Markers in the Federally Endangered, *Liatris helleri* (Asteraceae). App. In Plant Sciences *Accepted and awaiting print

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359–361

Frichot E, François O (2015) LEA : An R package for landscape and ecological association studies. Methods in Ecology and Evolution 6:925–929

François O (2016) Running Structure-like Population Genetic Analyses with R. R Tutorials in Population Genetics, U. Grenoble-Alpes. pp. 1-9

Gaiser LO (1946) The Genus Liatris. Rhodroa 48: 31–382

Gaiser LO (1950) Chromosome Numbers in Liatris. II. Graminifoliae and Pauciflorae. American Journal of Botany 37: 414

Godt MJW, Hamrick JL (1996) Genetic diversity and morphological differentiation in Liatris helleri (Asteraceae), a threatened plant species. Biodiversity and Conservation 5:461–471

Goudet J (2005) hierfstat, a package for r to compute and test hierarchical F-statistics. Molecular Ecology Notes 5:84–186

Hale ML, Burg TM, Steeves TE (2012) Sampling for Microsatellite-Based Population Genetic Studies: 25 to 30 Individuals per Population Is Enough to Accurately Estimate Allele Frequencies. PLoS ONE, 7(9) e45170. https://doi.org/10.1371/journal.pone.0045170

Hedrick PW (2005) A STANDARDIZED GENETIC DIFFERENTIATION MEASURE. Evolution 59(8): 1633–1638 https://doi.org/10.1111/j.0014-3820.2005.tb01814.x

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genetics 11: 94

Kamvar ZN, Tabima JF, Grünwald NJ (2014) Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:281

Levin DA (1967) An Analysis of Hybridization in Liatris. Brittonia 19:248

Kearse M. et al. (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

Medve RJ (1987) The Effects of Fire on Resource Allocation and Growth of Liatris spicata. American Midland Naturalist 117: 99

Murdock N (2000) Recovery Plant for Liatris helleri, Heller's Blazing Star. US Fish and Wildlife Service.

Nesom G (2005) Broadened Concept of LIATRIS HELLERI (ASTERACEAE: EUPATORIEAE). SIDA 21: 1323–1333

North Carolina Natural Heritage (NCNHP) (2019) Element Occurence Report for *Liatris helleri.*

Paradis E, Claude J, trimmer K (2004) APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics 20: 289–290

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28: 2537–2539

Prichard J, Stephens M, Donnelly P (2000) Inference of Population Structure Using Multilocus Genotype Data. GENETICS 155(2): 945–959

RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

Ramasamy R, Ramasamy, S, Bindroo B, Naik V (2014) STRUCTURE PLOT: a program for drawing elegant STRUCTURE bar plots in user friendly interface. SpringerPlus 3:431

Sutter RD, Benjamin SE, Murdock M, Teague B (1993) Monitoring the Effectiveness of a Boardwalk at Protecting a Low Heath Bald in the Southern Appalachians. Natural Areas Journal 13(4): 250-255

Takezaki N. Nei M (1996) Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 144: 389–399

Ulrey C, Quintana-Ascencio PF, Kauffman G, Smith AB & Menges ES (2016) Life at the top: Long-term demography, microclimatic refugia, and responses to climate change for a high-elevation southern Appalachian endemic plant. Biological Conservation 200, 80–92

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shipley, P (2004) micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4, 535–538

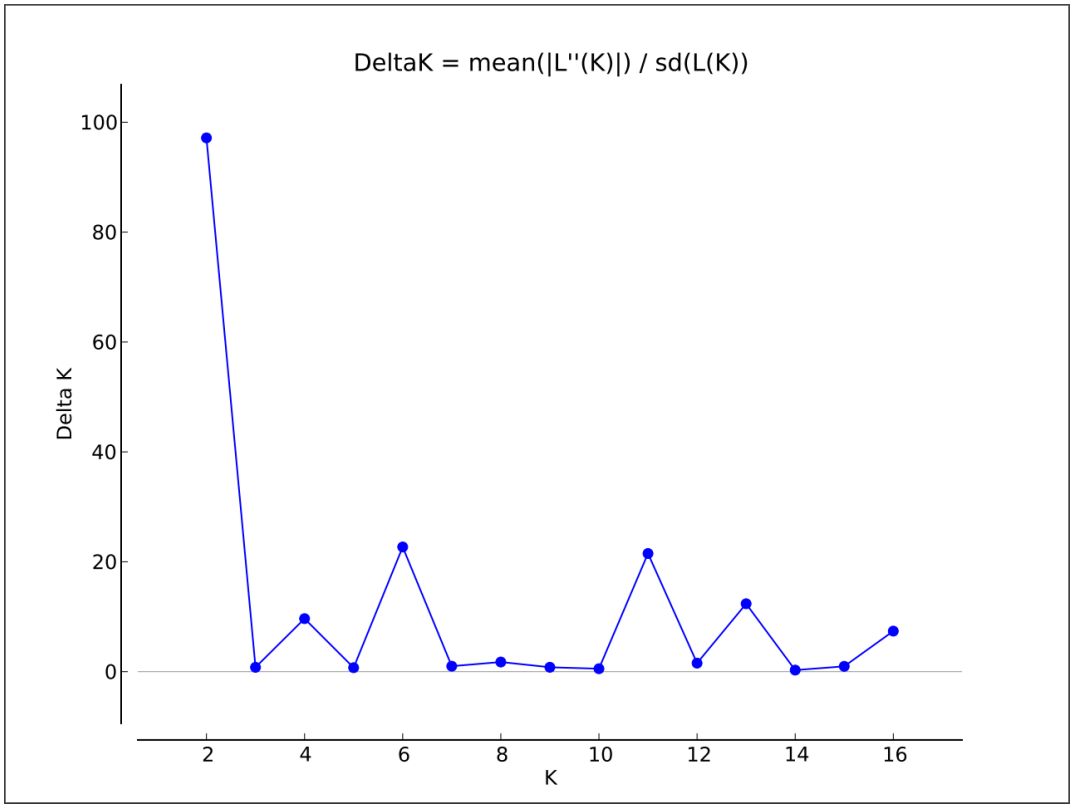
Vieira MLC, Santini L, Diniz AL, Munhoz CF (2016) Microsatellite markers: what they mean and why they are so useful. Genetics and Molecular Biology 39: 312–328

Weakley A (2015) Flora of the Southern and Mid-Atlantic States. University of North Carolina Herbarium, Chapel Hill, North Carolina, USA

Winter DJ (2012) MMOD: an R library for the calculation of population differentiation statistics. Molecular Ecology Resources 12(6): 1158–1160. https://doi.org/10.1111/j.1755-0998.2012.03174.x

Wiser S, Peet R, Peter W (1996) High-elevation rock outcrop vegetation of the Southern Appalachian Mountains. Journal of Vegetation Science 7: 703–722.

Appendix



A1 Delta K values generated using the Evanno Method in STRUCTURE HARVESTER. Indicates likely K values of 2, 4, 6, 11, and 13.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	
A	0	0.272	0.438	0.458	0.367	0.457	0.505	0.518	0.538	0.571	0.613	0.546	0.482	0.382	0.336	0.490	0.528	0.486	0.326	0.428	0.526	**A**
B	0.000	0	0.368	0.329	0.314	0.411	0.393	0.484	0.525	0.601	0.592	0.554	0.412	0.298	0.211	0.444	0.480	0.481	0.253	0.396	0.526	**B**
C	0.457	0.268	0	0.469	0.357	0.349	0.582	0.640	0.644	0.659	0.707	0.577	0.452	0.394	0.393	0.580	0.723	0.519	0.399	0.457	0.569	**C**
D	0.311	0.191	0.389	0	0.333	0.390	0.510	0.632	0.651	0.689	0.698	0.559	0.572	0.530	0.506	0.641	0.697	0.566	0.512	0.600	0.599	**D**
E	0.329	0.214	0.501	0.000	0	0.253	0.289	0.385	0.551	0.483	0.519	0.205	0.375	0.368	0.339	0.435	0.580	0.496	0.392	0.461	0.520	**E**
F	0.059	0.000	0.230	0.094	0.117	0	0.318	0.453	0.571	0.571	0.630	0.452	0.365	0.365	0.417	0.522	0.623	0.477	0.501	0.504	0.508	**F**
G	0.205	0.101	0.348	0.000	0.000	0.014	0	0.167	0.451	0.443	0.545	0.435	0.434	0.431	0.431	0.483	0.550	0.564	0.535	0.544	0.559	**G**
H	0.254	0.123	0.364	0.000	0.000	0.021	0.000	0	0.277	0.283	0.469	0.406	0.451	0.382	0.380	0.431	0.484	0.558	0.507	0.489	0.598	**H**
I	0.370	0.235	0.521	0.000	0.000	0.141	0.021	0.015	0	0.422	0.586	0.657	0.411	0.368	0.367	0.520	0.613	0.578	0.456	0.429	0.562	**I**
J	0.283	0.166	0.449	0.062	0.064	0.113	0.059	0.031	0.022	0	0.562	0.519	0.577	0.503	0.521	0.553	0.662	0.635	0.612	0.632	0.686	**J**
K	0.262	0.153	0.374	0.075	0.120	0.097	0.060	0.067	0.040	0.085	0	0.571	0.574	0.486	0.484	0.398	0.738	0.604	0.633	0.608	0.704	**K**
L	0.041	0.000	0.203	0.207	0.278	0.000	0.100	0.134	0.328	0.230	0.198	0	0.607	0.524	0.506	0.563	0.626	0.663	0.545	0.608	0.643	**L**
M	0.393	0.261	0.534	0.000	0.000	0.159	0.025	0.008	0.000	0.028	0.096	0.344	0	0.095	0.133	0.266	0.684	0.532	0.399	0.336	0.539	**M**
N	0.576	0.440	0.637	0.046	0.078	0.309	0.161	0.168	0.065	0.160	0.194	0.539	0.021	0	-0.007	0.132	0.587	0.471	0.251	0.253	0.518	**N**
O	0.587	0.419	0.572	0.045	0.089	0.294	0.160	0.154	0.151	0.170	0.234	0.509	0.089	0.025	0	0.176	0.487	0.463	0.138	0.179	0.524	**O**
P	0.512	0.257	0.441	0.000	0.000	0.131	0.000	0.000	0.000	0.000	0.050	0.359	0.000	0.000	0.000	0	0.739	0.648	0.423	0.453	0.707	**P**
Q	0.302	0.146	0.379	0.240	0.314	0.136	0.209	0.202	0.306	0.080	0.216	0.195	0.310	0.524	0.485	0.274	0	0.588	0.471	0.541	0.634	**Q**
R	0.421	0.295	0.449	0.000	0.046	0.183	0.038	0.027	0.035	0.096	0.117	0.320	0.026	0.045	0.039	0.000	0.295	0	0.503	0.506	0.529	**R**
S	0.111	0.000	0.167	0.262	0.311	0.050	0.194	0.203	0.328	0.225	0.246	0.017	0.344	0.491	0.441	0.273	0.100	0.344	0	0.283	0.535	**S**
T	0.283	0.153	0.348	0.014	0.046	0.093	0.035	0.035	0.000	0.047	0.000	0.201	0.025	0.083	0.089	0.000	0.166	0.045	0.207	0	0.424	**T**
U	0.117	0.044	0.388	0.081	0.077	0.037	0.039	0.038	0.040	0.010	0.028	0.114	0.068	0.218	0.242	0.047	0.070	0.149	0.132	0.019	0	**U**
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	

A2 Pairwise R_{ST} values between populations below diagonal. Pairwise G'_{ST} (Hedrick 2005) values between populations above diagonal.

Addendum: Future Directions and Closing Thoughts

Microsatellite Markers

The 17 microsatellite markers developed in this study were used to provide a high-resolution examination of population level dynamics in this system. These markers were also tested for their ability to cross amplify across the genus *Liatris*, so that this tool kit can be used to address other questions in the genus. Of the 17 markers developed, 11 provided successful amplification across 100% of tested individuals (Chp 2. Table 3). These markers will be the best choice for future studies within the genus, until higher level sequencing technology advances, by providing a tool to help delineate more definitive species boundaries throughout the genus, which will likely lead to more species in the genus by revealing cryptic species across the landscape. These cryptic species may not all warrant full species status, but possibly varietal or subspecies treatments of existing concepts.

Separation of Taxa

Based on these data, there is little doubt that a distinct group of populations within our current concept of *L. helleri* exists on the landscape. The distinct group of populations that were consistently identified as *L. helleri* would meet the criteria of the Phylogenetic Species Concept, defined as the smallest aggregation of populations or lineages diagnosable by a unique combination of character states (Wheeler & Meier, 2000). Thus, there should be no argument that this species, or at least this grouping of populations, should remain protected under the Endangered Species Act, which aims to target the most critical unit in need of conservation (Haig et al., 2006). The central argument in this system should be one of taxonomic nomenclature, not federal protection. It is my opinion that those populations identified in the STRUCTURE analysis of K=2 as retaining the highest percentage of *L. helleri* ancestry should be considered true *Liatris helleri* Porter. Those populations displaying predominantly *L. turgida* ancestry in K=2 should be treated as *Liatris turgida* Gaiser. The admixed zones in Burke County and Avery County should remain protected, and almost all currently occur on

protected land. These admixed populations may indeed represent those populations with the highest adaptive potential and may therefore be the most likely to adapt to a changing climate. These populations do still retain *L. helleri* ancestry and are likely to remain stable. The STRUCTURE analysis of K=4 also revealed structuring within *L. turgida* populations while maintaining the same distinct population grouping of *L. helleri*. This sub-structuring revealed distinct grouping in Burke County and Ashe County, which could be viewed as evolutionary significant units of conservation for *L. turgida*. The resulting genetic relation of these taxa makes logical sense in a geographic context. There appears to be pocket of distinct populations of *L. helleri* with hybrid zones on either side of the "pocket" and more "pure" populations of *L. turgida* on the periphery. Further sampling of *L. turgida* would likely reveal further population structure of this species. The protective status of *L. turgida* likely needs to be reevaluated throughout its range, but at least in the state of North Carolina. During the field season of 2018 this was not a commonly observed species and warrants additional monitoring.

The separation of these taxa could also be viewed on a subspecies level. Kapadia (1963) defines a subspecies as partial populations (e.g. natural groups) of the same general nature as the species but exhibiting a lower degree of morphological differences and/or reproductive isolation. This concept of subspecies would seem to fit nicely to the results observed in this study. This group of populations along the Blue Ridge Parkway are no doubt distinct, but currently lack diagnosable morphological characters and also seem to be experiencing reproductive isolation. This may potentially warrant these populations to be considered *Liatris helleri* subsp. *helleri* and those populations considered *L. turgida* to be subsumed under the general *helleri* epithet.

Distribution and Rarity

Does the grouping of distinct *L. helleri* populations represent an ancestral relict of a more widespread metapopulation or species? Is this species naturally rare, or has its rarity been induced by anthropogenic activity? The higher observed allelic diversity in this species could lend evidence to

this hypothesis. It is likely that *L. helleri* does indeed represent a post-Pleistocene relic like several of the other species found in the high elevation rock outcrop communities of the southern Appalachians (Ulrey et al., 2016; Wiser et al., 1996). Gaiser (1946) also hypothesized the southern highlands as the center of origin for the genus *Liatris* given its high diversity of forms in the southeast. In this scenario it would be likely that the group of populations identified in this study represent an ancestral population, which would further increase their conservation value. Given this specie's proclivity toward high elevation rock outcrop communities, it may be naturally rare and limited by available habitat. However, the connectivity and size of the extant populations has undoubtedly been negatively impacted by human activity.

Management Recommendations

The population grouping that represents those populations of *L. helleri* along the Blue Ridge Parkway should receive the bulk of resources allocated to the conservation of this species. These populations contain few individuals (~15-50) and are subject to some of the highest recreation intensity effects, such as trampling and harvesting. Measures to increase education about this species and its natural community need to be taken in order to make visitors more aware of their impacts and hopefully more deliberate in their actions. Recreation continues to threaten these habitats through trampling, soil degradation, poaching, and introduction of invasive species. A study at one of the most popular sites along the Blue Ridge Parkway saw significant vegetation declines, even after the installation of a boardwalk to limit trampling (Sutter et al., 1993). Public outreach, increased signage, and direct monitoring during peak visitation months could all potentially alleviate direct pressures from recreation. Limiting access to certain populations is never ideal on public lands, but also may serve as a more viable option for protecting these populations.

Augmentation attempts in the past have been successful (NCNHP, 2019) and could prove a useful tool in the management of this species in order to increase numbers of individuals within populations. Care should be taken to ensure augmented plants are sourced from the local population

in order to maintain the integrity of this distinct gene pool and avoid the loss of local adaptation. This species grows well in a greenhouse setting, but previous attempts grew plants in 1 quart pots, which facilitated the growth of a thick corm. Using this method, the corm was sometimes too bulky to be planted in this species' shallow soiled habitat (R. Lance, per comm.). Future efforts to cultivate this species in a greenhouse setting should grow plants in shallow trays or pots in order for a shallower corm to form, which would allow for more successful outplanting into a natural setting.

Demographic work in this species has been also carried out across several populations within and outside of the distinct grouping identified through this study (C. Ulrey, per. comm.). This work should be continued into the future for both taxa as it could likely help elucidate long-term trends and demographic differences between these taxa as well as model future trends for these populations. Efforts should be made to standardize demographic data collection in order to have a cohesive dataset to allow comparisons over time and between localities.

In order to provide taxonomists and land managers with an even more comprehensive concept of this species, ecological studies may provide more resolution. By examining the ecological parameters of those populations identified as retaining predominately *L. helleri* ancestry vs. those of *L. turgida*, we may be able to elucidate abiotic and biotic properties that separate these taxa ecologically. These include soil profiles of: nutrients, bacteria, and fungi, photosynthetic and other physiological parameters, percent canopy cover and light exposure, aspect and elevation, as well as wind currents driving seed dispersal. As these taxa inhabit distinct natural communities with unique plant assemblages there may be an underlying ecological driver of this separation. The ecological separation in conjunction with geographic separation via distance is likely facilitating the isolation of the small group of populations identified to retain distinct *L. helleri* ancestry from those populations of *L. turgida*. Future morphological work should also be utilized to investigate those populations identified with the highest *L. helleri* ancestry to determine if there is indeed any morphological separation of these taxa. This approach should first address pappus length, as this was the previous

distinguishing character, but micromorphology of seeds and other fine scale features may also prove useful in infrageneric classifcation.

During the field season of 2018 I did not observe any distinct morphological or ecological separation. There were plants in Virginia growing on slightly lower elevation outcrops that shared a similar short and stocky morphology as well as similar habitat to more southern populations in North Carolina. Most populations of *L. turgida* were slightly taller, around 1m (taller than the key for *L. helleri*) and possibly a slightly later phenology with many populations of *L. turgida* visited at lower elevations displayed a slightly delayed phenology, sometimes not flowering until mid-August, which would be concurrent with previous classification of these taxa. If there are any quantifiable ecological parameters separating these taxa they could operate as secondary criterion for the taxonomic delineation between them.

The most striking feature of the results was that those populations identified as retaining the highest percentage of *L. helleri* ancestry are primarily located within a small geographic area known as the Boone Fork Bowl. This area along the approximately southeast facing slope of Grandfather Mountain occurs around 4000 +/- feet in elevation and may provide some sort of shielding effect via up drafts of wind or provide a highly specific niche. There appears to be populations of admixture along both ends of Grandfather Mountain and even a minor amount on top, possibly due to augmentations, but somehow this “pocket” of populations or metapopulation has remained isolated. The area above this bowl was previously used for hang gliding, so maybe there is a sort of thermal draft driving wind up the mountain allowing for dispersal out of the bowl, but not into, the bowl. Future work should investigate the ecological dynamics of this area, which may shed light as to how this metapopulation has remained relatively isolated.

References:

Gaiser, L. O. (1946). The Genus Liatris. *Rhodroa, 48*(575), 331–382.

Haig, S. M., Beever, E. A., Chambers, S. M., Draheim, H. M., Dugger, B. D., Dunham, S., … Sheffield, L. M. (2006). Taxonomic Considerations in Listing Subspecies Under the U.S. Endangered Species Act. *Conservation Biology, 20,* 1584–1594. https://doi.org/10.1111/j.1523-1739.2006.00530.x

Kapadia, Z. (1963). Varietas and Subspecies, a Suggestion Towards Greater Uniformity. *Taxon*, *12*, 257–259.

North Carolina Natural Heritage (NCNHP) (2019) Element Occurence Report for *Liatris helleri.*

Sutter, R.D., Benjamin, S.E., Murdock, M., Teague, B. (1993) Monitoring the Effectiveness of a Boardwalk at Protecting a Low Heath Bald in the Southern Appalachians. *Natural Areas Journal* 13: 250-255

Ulrey, C., Quintana-Ascencio, P. F., Kauffman, G., Smith, A. B., & Menges, E. S. (2016). Life at the top: Long-term demography, microclimatic refugia, and responses to climate change for a high-elevation southern Appalachian endemic plant. *Biological Conservation, 200,* 80–92. https://doi.org/10.1016/j.biocon.2016.05.028

Wheeler, Q. D., & Meier, R. (Eds.). (2000). *Species concepts and phylogenetic theory: a debate*. New York: Columbia University Press

Wiser, S., Peet, R., & Peter, W. (1996). High-elevation rock outcrop vegetation of the Southern Appalachian Mountains. *Journal of Vegetation Science, 7,* 703–722.

Vita

Logan Crawford Clark was born in Ahoskie, North Carolina in 1995 to Shirley and Jason Clark. He came to Boone, NC in 2013 to attend Appalachian State University where he received a Bachelor of Science in Ecology, Evolution, and Environmental Science in the spring of 2017. During the following fall of 2017 he started his graduate research at Appalachian to pursue a Master of Science in Biology. Upon receiving his MS degree in the spring of 2019 he plans to continue to pursue work in the fields of conservation and land management.